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SYMPOSIA OF THE SECTION ON MICROBIOLOGY

THE NEW YORK ACADEMY OF MEDICINE

Number 5

THE NATURE AND SIGNIFICANCE OF THE ANTIBODY RESPONSE

THE NATURE AND SIGNIFICANCE OF THE ANTIBODY RESPONSE

EDITED BY

A. M. Pappenheimer, Jr.

SYMPOSIUM HELD AT THE
NEW YORK ACADEMY OF MEDICINE
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Preface

In arranging this symposium an effort was made to emphasize antigen-antibody reactions in animal tissues, with considerable stress on studies of antibody formation and antigen-antibody reactions in man himself. There was nothing very modest about the program and the reader will find that the great gaps in our knowledge are only too apparent. One positive contribution I feel confident the symposium does make: that is to demonstrate how fruitful the application of *quantitative* immunochemical methods can be in throwing light on the mechanisms of immunological reactions. It is nearly twenty years since Heidelberger and Kendall devised simple but elegant methods for quantitative estimation of small amounts of antigen and antibody. Yet it seems to me that the chemists were the first to comprehend the usefulness of these methods—most of the early applications were in the fields of protein and polysaccharide chemistry. It is only quite recently that others have begun to use the methods to solve problems in the field of immunology itself. It is becoming more and more apparent that such baffling problems as those encountered in the hypersensitive states can only be elucidated by intelligent application of quantitative methods. This meeting should be looked upon as a tribute to Dr. Michael Heidelberger and his associates, since without their work this present symposium would not be possible.

In preparing the symposium for publication, the order of the papers as originally presented has been altered somewhat so as to provide greater continuity. Chapters 1–5 deal with Production of Antibodies, Chapters 6–9 with Kinds of Antibodies, and the final Chapters concern Antigen-Antibody Reactions in the Tissues. The discussion which followed each group of papers, though not extensive, was pertinent and has been included.

A. M. Pappenheimer, Jr.

New York University College of Medicine
May 8, 1951

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THE NATURE AND SIGNIFICANCE OF THE ANTIBODY RESPONSE

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Chapter 1

THEORIES OF ANTIBODY FORMATION[1]

By Felix Haurowitz, *Indiana University*

Immunological response is essentially antibody formation; it is evident, therefore, that we cannot understand this response unless we understand antibody formation. The fact that there is more than one theory of antibody formation shows that none of the existing theories is fully satisfactory. Before discussing critically these theories it will be useful to define the terms antigen, antibody, and enzyme, used throughout this discussion.

Antigens, according to the usual definition, are substances which on parenteral injection cause the production of antibodies in the animal body. Since it is generally accepted at present that antibodies are proteins, we can define antibodies as proteins which (a) are formed in the injected organism instead of normal proteins, and (b) react specifically with the injected antigen.

The necessity to define enzymes in this discussion arises from the fact that antigen, antibody, as well as complement have been considered as enzymes by different authors. Enzymes are macromolecular catalysts of biological origin, and catalysts, according to the usual definition, are substances which increase the velocity of chemical reactions or render these reactions possible without being used up in the reaction. Since antigens induce the formation of antibodies, they have been considered as catalysts. The enzyme character of antigens becomes problematic, however, when we use the more rigorous thermodynamic definition of catalysts. According to this definition, catalysts lower the activation energy of chemical reactions. The activation energy, E_a, is the energy required to raise the energy content of the reactants to that of the reactive "transition state." The activation energy can be determined by measuring the velocity constants, k_1 and k_2, of the reaction at two different temperatures, T_1 and T_2. The activation energy in calories per mole is

[1] This investigation was supported in part by a research grant from the National Institute of Health, United States Public Health Service.

$$E_a = R \frac{T_1 T_2}{T_2 - T_1} \ln (k_2/k_1),$$

where R is the gas constant (1.98 cal. per mole) and T the absolute temperature. In the presence of catalysts the ratio k_2/k_1 becomes smaller, so that E_a decreases. There is no indication that antigens influence the ratio k_2/k_1 of protein formation at two different temperatures. Accordingly, antigens will not be considered as catalysts in this discussion.

The first theory of antibody formation was advanced by Paul Ehrlich (1). He was of the opinion that traces of each type of antibody are preformed in the organism as so-called receptors and that a receptor is regenerated at an increased rate when the corresponding antigen is injected. This view had to be abandoned when Landsteiner (2) showed that the organism is able to produce antibodies against artificial products of the chemical laboratory, such as arsanilic or tartranilic acid. It is unimaginable that the organism of an animal contains preformed receptors against such synthetic chemicals. Although the hypothesis of preformed receptors has to be rejected, another concept of Ehrlich's theory is essentially correct. Ehrlich for the first time suggested complementariness of antigen and antibody particles. While it was believed at that time that the antigenic action is due to the whole antigen particle or to the whole antigen molecule, it was proved by Landsteiner that the specificity of the antigen is determined by small chemical groups; we call them determinant groups (3, 4). It was believed for a time that these groups, or similar groups produced in the organism, are incorporated into the antibody molecule (5, 6). This is certainly not the case, because the number of antibody molecules formed exceeds by far the number of antigen molecules injected (7, 8). Moreover, antibodies against the determinant azo-phenyl-arsonic acid group were found to be free of arsenic, and antibodies against iodinated proteins free of iodine, even when the injected animals were supplied with sufficient quantities of iodine (9).

It has been known for a long time that the soluble antibodies of the immune serum are found in the globulin fraction. In 1930 Breinl and I (10) advanced the hypothesis that the globulins *are* the true antibodies and that their antibody function is due to the complementary adaptation of their shape to the shape of the determinant group of the antigen. A similar view was advanced, independently and almost at the same time, by Alexander (11) and by Mudd (12). This concept of the

nature of antibodies is probably too narrow. Possibly the insoluble proteins of the connective tissue and of the smooth muscles also react specifically with the antigen. Therefore the original view on the nature of antibodies was extended to include also proteins of other than the serum globulin type (13). However, it is very difficult to analyze quantitatively the reactions of the insoluble "fixed" antibodies. Therefore the present discussion will be limited to antibodies of the serum globulin type.

The view that antibodies are identical with certain serum globulins, and are not other substances carried by the globulins, is supported by the fact that all properties of purified antibodies are the same as those of the normal serum globulins. This is valid not only for the amino acid composition of the antibodies, but also for their molecular weight, their isoelectric point and other physical-chemical properties, although slight differences between antibodies and normal globulins have been found. However, similar differences exist between the various globulin fractions of a normal serum (14).

Obviously, there must be some differences between antibodies and normal serum globulins. Since all known determinant groups of the antigen molecules are polar groups, it was assumed that their electrostatic forces direct the amino acid precursors of the protein into definite positions, so that the protein formed would be adapted in a manner complementary to the determinant group of the antigen. This could be achieved by changes in the sequence of amino acids in the peptide chain, or by a different mode of folding of the peptide chain. Pauling, accepting our complementariness hypothesis, advanced the view (15) that the difference between normal globulins and antibodies was a difference in the mode of folding of the peptide chains and that the sequence of the amino acids was probably the same in both antibodies and normal globulins. This very plausible view is supported by recent analyses of R. R. Porter (16), who found alanyl-leucyl-valyl-aspartic acid as the terminal residue in anti-ovalbumin from rabbit serum and also in the inactive gamma globulins of normal rabbit serum.

If antibodies differ from normal globulins only by the mode of folding of their peptide chains, one could imagine that normal globulins are converted into antibodies by unfolding and refolding of their peptide chain (17). Such a conversion is improbable, since it is clear from experiments with isotopic nitrogen that antibodies are formed in the

organism at the same rate as normal serum globulins and that there is no exchange of isotopic nitrogen between active antibodies and the normal serum globulins (18). Moreover, attempts to manufacture antibodies *in vitro* by the cautious denaturation of normal globulins in the presence of antigen have failed (19, 20). All this demonstrates that antibodies are not formed by a rearrangement of normal globulin molecules but are formed by the action of intermolecular forces of adjacent antigen molecules upon the precursors of normal globulin molecules.

Before we accept this view we have to examine the objections raised against it. The main opponent against a direct role of the antigen in antibody synthesis is Burnet, who recently presented his views in a stimulating book published with Fenner (21). Burnet believes that antibody formation is started by the antigen, but that it can go on after the disappearance of all of the antigen from the organism. This view is based chiefly on the long persistence of immunity after infection with yellow fever virus and other viruses. Burnet and Fenner state that it is "impossible to think . . . of virus being retained as antigenic pattern for so long" (21). However, the same authors admit the long persistence of the lymphocytic choriomeningitis virus in mice and attribute it to symbiosis (21). Could not the long-lasting immunity against yellow fever and other virus diseases be due to the symbiosis with a mitigated noninfective virus? (2). Obviously, no symbiosis is possible if we are dealing with lifeless antigens such as antigenic proteins or polysaccharides. However, during the last few years, Felton (22), Coons (23), and McMaster and Kruse (24) have proved that bacterial polysaccharides are stored in the organism of mice for many months. Burnet considers these cases as exceptional, since he is chiefly interested in particulate antigens such as cells, bacteria, or viruses. There is no doubt that particulate antigens are broken down in the organism and that they become invisible. However, this does not mean that the true antigenic agent—the determinant group of the antigen molecule—disappears.

In order to explain the formation of antibodies in the absence of antigens, Burnet assumes that the expendable body cells contain so-called self-marker components which make them recognizable to the cellular enzymes. Thus the blood group substance A is the self-marker of the erythrocytes in persons of the A group. If an antigen similar to the self-marker (for example, blood group substance B) is injected, the enzyme unit whose function it is to destroy the self-marker will be adapted to

the antigen. Burnet assumes that these enzymes reproduce themselves, so that the formation of adapted enzymes goes on after the disappearance of the antigen. The circulating antibodies, according to Burnet, are partial replicas of these adapted enzyme units; they are adapted to the antigen, but devoid of enzymatic activity and of the ability of self-duplication.

Burnet and Fenner's theory has been criticized severely by its own proponents. Burnet and Fenner admit that their view goes far beyond classical chemistry, that there is no evidence for enzyme adaptation in other fields, and that we do not know of any enzymes possessing the type of specificity shown by antibodies. Burnet and Fenner base their belief in enzyme action on the exponential increase of the antibody titer in the immune serum. This, in the authors' words, means that "something somewhere is proliferating in true biological fashion." According to Burnet and Fenner, antibody production is not only a function of the cells originally stimulated but also of their descendants. This is in discordance with the experience that acquired immunity is never transmitted to descendants. Moreover, Burnet's idea of preformed enzymes shares with Ehrlich's theory of preformed receptors the inability to explain the formation of antibodies against those artificial determinant groups which are quite different from any "self-marker" component.

Burnet's views have been discussed here at some length because we cannot understand antibody formation unless we know whether or not antibody can be formed in the absence of the determinant group of the antigen. It is very difficult to prove analytically the presence or the complete absence of small amounts of the antigen or of its determinant group, because we lack sufficiently sensitive test methods. However, the view that the presence of antigen is necessary for antibody synthesis is supported by the finding of bacterial polysaccharides in the tissues of mice many months after their injection (22, 23, 24). We are entitled, therefore, to speculate about the possible role of the antigen in the mechanism of antibody formation.

Since antibodies are proteins we have to ask how proteins such as the serum globulins are formed in a normal organism. The serum globulins are more or less ellipsoidal molecules; the length of their axes varies from 40 to 700 Angström units. Direct replication of such particles would require long-range forces operating over distances of at least 50–100 Angström units. Since we do not know of such forces we have to assume

that the replication of proteins occurs in an expanded thin protein film, and that this film, secondarily, is folded to form the globular protein molecule. Accordingly the process of protein synthesis is a two-phase process.

In the first phase, free amino acids or small peptide fragments combine to form the long peptide chain of the protein film. Obviously an enzyme is necessary to overcome the considerable activation energy required for peptide bond formation. However, the specific order of the amino acids in the peptide chain cannot be attributed to the action of an enzyme; for we do not know of any enzyme whose specific action extends beyond a localized atomic group. The existence of species-specific enzymes has been claimed by Abderhalden (25). Despite many attempts we have not been able to confirm his results and to find proteolytic enzymes splitting preferentially the parenterally administered foreign protein (26). Enzymes, like other catalysts, act by means of their active group, and it is evident that such a group cannot "arrange" a definite sequence of the amino acids. A better explanation of the specific order of the amino acids in a peptide chain is gained when we assume the existence of an identical peptide chain as an "organizer." Its action would be similar to that of a crystal seed in crystal growth. The analogy between crystal growth and biological replication has been emphasized repeatedly by Troland (27), H. J. Muller (28), Haldane (29), and others (30). The phenomenon of crystal growth demonstrates clearly that dissolved molecules are preferentially attracted to molecules of the type that form the solid crystal. We can assume, also, that amino acids dissolved in the cellular fluid will be attached preferentially to similar amino acid residues of the peptide chain which acts as an organizer, so that, for example, alanine, tyrosine, or serine will be attached to the alanyl, tyrosyl, or seryl residues of the organizer molecule. In this manner the amino acids would be lined up in the specific order of the organizer molecule. In the presence of a non-specific peptidase this chain of free amino acids attached to the organizer will undergo conversion into a peptide chain containing these amino acids in exactly the same order as the organizer molecules (31). In addition to the formation of peptide bonds between amino acids, transpeptidation (32) may play an important role in the formation of the long peptide chains.

The second phase of protein formation is the folding of the replica film—its conversion into a globular protein molecule. For obvious

reasons we have to postulate that the organizer molecule does not fold. Most likely it is kept in the expanded state by combination with nucleic acids and formation of an insoluble nucleoprotein film (31).

Since identical peptide chains are present in normal globulins and in antibody molecules, we assume that the antigen does not interfere in the first phase of protein synthesis. Its action is apparently restricted to the second phase, the formation of the three-dimensional protein molecule from the peptide film. The folding evidently occurs in such a manner that the globulin formed is complementarily adapted to the determinant group of the antigen molecule (31). This assumption is based on the polar properties of determinant groups. We know that polar groups act on adjacent atoms and molecules by means of their electrostatic forces. There is no indication for any catalytic action of the antigen in this process.

It is difficult to speculate whether the globular particle finally formed will have one, two, or more complementarily shaped groups. Considering the dilution of antigen molecules in the organism, it is reasonable to assume that only one of them will be present at the site of the synthesis of a protein molecule, and that each of the antibody molecules will have only one complementary group. Pauling (15) and other authors prefer, however, to consider the precipitating and agglutinating antibodies as bivalent or multivalent.

Since all cells produce proteins we may ask whether all of them produce antibodies. This is probably the case, provided the cell can trap the antigen and store it without destroying it. Therefore antibody production is not restricted to the reticulo-endithelial or the lymphatic system, but occurs even in the nervous system (33) or in the cornea (34) as has been shown recently. We cannot expect that all antibody molecules of an immune serum are identical. Their complementary groups may be formed by different parts of the antibody molecules. They may be adapted to different determinant groups of an antigen. Moreover, their degree of adaptation may be more or less imperfect. Actually we find in each immune serum a multiplicity of antibodies (2, 35, 36, 37). Most likely there exist many intermediates between normal serum globulins and the well-adapted antibody molecules (38, 39, 40).

From the foregoing discussion it is evident that we consider antibody formation essentially as protein synthesis in the presence of antigen. One may ask, what happens when no antigen is present. Probably some

normal cellular constituent has then the role of a template, so that the shape of the protein formed will be complementary to the determinant group of this normal template. Accordingly, the normal globulins have been designated as "autoantibodies" (41).

Since acquired immunity and antibody formation are not inherited it would be interesting to know whether antibodies are formed in the nucleus or in the cytoplasm. When iodo-proteins labeled with I^{131} were injected intravenously into rabbits, most of the antigen found in the liver appeared at first in the microsome fraction (42); however, within several minutes the bulk of the antigen was encountered in the mitochondria, although a considerable proportion was also found in the nuclei (43). The finding of most of the antigen in the mitochondria is particularly interesting because these subcellular granules are endowed with the property of self-duplication (44), that is, of protein synthesis. Possibly these are the antibody producers.

SUMMARY

Antibody formation is considered as a modification of the normal formation of proteins. Protein synthesis is probably a two-phase process. In the first phase, an expanded peptide film is converted into the three-dimensional protein molecule. The antigen interferes only in the second phase, so that the globular protein molecule becomes complementarily adapted to the determinant group of the antigen.

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Chapter 2

SITES OF ANTIBODY FORMATION

By Philip D. McMaster, *The Rockefeller Institute for Medical Research*

Far too little is known about the sites of antibody formation and even less about the mechanisms involved. Nevertheless much good work has been done, and so many papers have been written that there seems to be a need for a brief impartial review of the trends of contemporary thinking about the subject. The present chapter constitutes such a review up to the beginning of the year 1951. For the sake of brevity no attempt will be made to trace fully the historical background of our present knowledge or to include in an exhaustive bibliography the names of all authors who have written on the subject. The latter would, of itself, be longer than the present chapter.

Instead there will be cited only very little of the older work and only part of the new, mostly readily accessible papers which have played a significant part in the development of present knowledge. Further, the attempt has been made to state impartially and as briefly as possible what we believe the authors themselves wished to convey. From the reference list an interested reader should be able to obtain all necessary references, but the review is presented especially for workers in other fields who desire a short survey of the present state of knowledge about the sites of antibody formation.

THE ROLE OF THE RETICULO-ENDOTHELIAL SYSTEM

Following the early work of Metschnikoff (1, 2), Wyssokowitsch (3), Pfeiffer and Marx (4, 5), the Wassermanns (6, 7), and others (8), it has been recognized that certain phagocytic cells of the tissues and endothelia—now collectively known as the reticulo-endothelial system of Aschoff (9)—are capable of seizing upon bacteria or other particulate antigenic substances. It was only natural for the earlier workers to surmise that the cells which take up foreign substances are also instrumental in forming antibodies. Since these cells are almost everywhere

throughout the body, almost every organ has been considered as the site of antibody formation. To mention but a few of these sites, authors have alleged, at one time or another, that antibodies are produced in the spleen, bone marrow, liver, lungs, lymph nodes, blood, or in the eye, in the central nervous system, in the skin, or in all of them.

The belief that reticulo-endothelial cells form antibody was bolstered by the finding that "blockade" of these cells by inert particulate matter (8–10) cut down antibody production. Today the interpretation put upon this finding by some is different: the factor cut down by "blockade" may be the capture and preparation of antigen rather than the production of antibody. Nevertheless it is interesting that the newer work has not yet shown that reticulo-endothelial cells either do not, or cannot, form antibody.

As these matters have been adequately reviewed (8, 10), mention needs to be made of only one recent paper. In 1939, Sabin (11) reported that an alum precipitate of a colored azoprotein of Heidelberger and Kendall (12, 13) could be recognized in phagocytic cells of the liver and omentum. These cells apparently took the material into digestive vacuoles and altered it by removal of the dye, after which the solid particles of protein disappeared and probably passed into the cytoplasm. Next the cells shed some of their cytoplasm—the macrophages, the big eaters, had become clasmatocytes, the shedders of cytoplasm. At the same time antibodies appeared in the blood. It was thought that from the engulfed material the cells formed globulin, some of it antibody which was liberated by the shedding.

In the same paper Sabin called attention to the take-up of the dye protein precipitate by great numbers of polymorphonuclear leucocytes which were, in turn, engulfed by the macrophages. The latter, therefore, obtained antigen directly and also after it had been acted upon by the leucocytes. Sabin recognizes the fact that the shedding of cytoplasm and simultaneous appearance of antibodies in the blood may be related only in time, and that the observed phenomena may have constituted a preparation of antigen for other cells.

LOCAL ANTIBODY FORMATION

Much work has indicated local antibody formation at the sites of antigen injection, but only a little is definitive.

Before discussing this work a few words of warning are essential. Physiologists (14–18) have shown that the blood vessels of injured and injected areas become more permeable than normal, and allow substances in the blood to localize in the injured tissues. As a result local high titers of antibody may mean only a seepage from the blood of antibody formed elsewhere in the body, and not formed where it is found. When authors claim a single cell type or a single organ as the site of antibody formation, one must inquire into the portal of entry of the antigen and the amount given. These, as will be seen below, are extremely important. To anticipate, if a little antigen is injected intradermally, the nearest lymph node will show more antibody than the blood or other organs, in the early stages of antibody production. By contrast, if larger quantities are introduced, antigen will pass the regional nodes and flood the body, calling into action other sites of antibody formation; then more antibody may be found in the blood, or spleen, or in various other localities. Again, small amounts of antigen injected into the blood may yield, in the first few days, more antibody in the spleen than in the serum, but when larger amounts are given, the opposite may be true—presumably other sites of antibody formation are called into action. Many other examples could be given.

Local Antibody Formation in the Eye. Several workers have injected various antigens into the eye—to compare the subsequent antibody titer in its tissues or fluids with that of the blood and other organs—among them Römer (19), Hektoen (20), Schamburow (21), Seegal and Seegal (22–25), Thompson (26, 27) and recently Thompson and Olsen (28). Most of them found higher titers in the blood than in aqueous humor or eye tissues. Schamburow, after injecting one eye, studied the titer in the other. He obtained higher titers in the blood of most of his instances, equal titers in others, but in some a higher titer in the uninjected eye than in the blood. Thompson and his colleagues (26, 27) and lately Thompson and Olsen (28) report the finding of precipitins in corneal tissues earlier and in higher concentration than in the serum, spleen, or bone marrow.

In contrast, the Seegals (29) have developed local hypersensitivity in rabbits' eyes unspecifically injured with glycerin at the same time that the animals received repeated intravenous injections of egg white. The observation is of importance, showing the localization of foreign materials in injured tissues leading to a subsequent hypersensitivity.

Antibody Formation in Skin. The skin has come in for a large share in the work upon local antibody formation, notably by Cannon and his associates, Pacheco, Sullivan, Walsh, and Hartley (30–32). They reported (31) a higher titer of antibody in vaccinated skin than in blood serum, liver, or spleen, following injections of antigens into areas in which large numbers of macrophages had been mobilized by multiple inoculations of the same organism. In one animal antibodies appeared in the skin before they did elsewhere. If the antigen was injected intravenously the titer was higher in the spleen and liver than in the skin. They suggest that there is a local formation and retention of immune bodies.

The problem has been attacked ingeniously by Hartley (32), who attempted to demonstrate local formation of anti-viral substances by skin. The phenomenon had already been reported, but not proven, by Orskov and Andersen (33). Hartley induced large numbers of macrophages, with very few lymphocytes or polymorphonuclears, to collect in skin injected with an aluminum hydroxide gel. The gel was capable of absorbing vaccine virus. When a virus-gel combination was inoculated into the tissues—where previous injections of gel had mobilized macrophages—neutralizing antibodies appeared before they did in undiluted blood or in extracts of other tissues. Since the virus was adsorbed on the gel, and the latter was held in the macrophages, Hartley suggests that the neutralizing antibodies were formed by the macrophages.

DeGara and Angevine (34) found antibodies at injected skin sites, but also in the spleen, bone marrow, liver, occasionally in lymph nodes, but not in the blood. They feel that repeated injections of antigen may mask the presence of antibody at injection sites, or in nodes, or wherever the antigen may reach. It seems to these authors that antibody formation occurs locally at the site of inflammation and in the organs that fix antigen, and that macrophages are responsible.

The important works of Freund and his co-workers (35–39) and of Westwater (40, 41), which are too complex to be reviewed, should be read to gain insight into the relationship of antibody to antigen in various organs, the blood to tissue ratio of antibodies, and the presence of antibodies at skin sites injected with killed tubercle bacilli in paraffin oil or other adjuvants. It is of much interest that the retention of antigen at the site of injection enhances antibody formation so greatly. The

mechanism is not well understood, but the work may soon lead to important findings.

The Respiratory Tract. Walsh and Cannon (42) studied the tissue to serum antibody ratios in rabbits injected intranasally or intraperitoneally. Having found them like those reported by Freund, after general immunization, they showed that intranasal injections of rabbits yielded a higher concentration of antibody in nasal tissue than in the blood. Since irritation of the nasal mucosa did not increase the tissue to blood antibody ratio it seemed as though the higher intranasal titer was not a simple concentration of antibody in an inflamed region. To test the point they simultaneously introduced one antigen into the nose and another into the peritoneum, and vice versa. The intranasal titer of antibody was high for the nasally introduced antigen, while the splenic titer was low. The reverse was true for the antigen introduced into the peritoneum, although the blood antibody titers to both antigens were often equal.

Antibodies in Tissue Culture. Tissue culture methods, which at first seemed to offer a simple means for spotting the cells that can elaborate antibodies, still leave much to be desired. The findings are too conflicting to warrant a review by any author who has not himself had long experience with the subject. Just the briefest mention of some of the work will be attempted here.

When animals have been injected with various antigens and tissues have been taken for culture later, antibody production has been reported by several workers (43–52) but not by others (53–56). It seems that tissues taken from normal animals and treated in culture with antigens have not as yet been enticed into making antibodies. This negative finding has important implications. In this connection the work of Parker is outstanding (43). Splenic tissue from rabbits, removed two or three days after injecting the animals with guinea pig cells, produced agglutinins in cultures. Spleens, taken one day after the injection, failed to do so, pointing to the existence of reactions within the organism that must reach a certain stage before antibody production can be completed. The serum did not contain the necessary reactants. For example, serum from the rabbits furnishing splenic tissues that formed agglutinins in cultures failed to bring about antibody formation when it was added to cultures of splenic tissue removed from other rabbits only one day

after injecting them with antigen. The tissue cultures of splenic plasma cells and lymphocytes by Fagraeus (45) will be discussed below. (For further papers, the reader may well turn to the bibliographies in references 43, 44, and 45.)

It is a matter of interest that Landsteiner and Parker (57) have shown that chick embryo fibroblasts in tissue culture, after 35 passages in rabbit plasma and rabbit embryo juice, were able to form proteins identical with or very closely related to chicken-serum proteins. Perhaps new techniques of the future will show that normal tissues *in vitro* can form antibodies.

Antibody Formation in the Spleen. There is so much well-known older work, some of it already mentioned, indicating antibody formation by the spleen—especially if antigen is intravenously injected—that lack of space forbids a full review here. Suffice it to say that as early as 1898 the work of Pfeiffer and Marx (4, 5), Wassermann (6), and Deutsch (58) indicated some sort of relationship between the spleen and antibody formation, as expressed by the presence of high titers in the organ after injecting various antigens. A few earlier workers, among them Jatta (59), found no such relationship, but other, later workers, among them Castellani (60), Tsurumi and Kohda (61), and Topley (62) disagreed.

The ghost that haunted most of the work was the unsolved problem: Is a high titer of antibody in an organ evidence of antibody formation, or is it simply a sign of localization after formation elsewhere? Since excellent reviews together with many references are to be had in the monographs of Perla and Marmorsten (63, 64), and since the subject of antibody take-up by tissues from the blood has been so ably discussed in the papers by Freund (35–39), nothing more needs to be added here.

Splenectomy. Removal of the spleen to study its effect upon antibody formation—done as early as 1892 by Tizzoni and Cattani (65), if not earlier by others, and in 1899 by Deutsch (58) and Roth (66), and later by Hektoen (67, 68), Luckhardt and Becht (69), and by Motohashi (70)—yielded better evidence that antibodies are formed in that organ. Motohashi was probably the first to point out that injections of small amounts of antigen bring out the antibody-forming function of the spleen, whereas large injections mask it.

Quite recently a few workers have added to our knowledge. Wolfe, Norton, Springer, Goodman, and Herrick (71) reduced precipitin forma-

tion in chickens by splenectomy—an observation not made before in non-mammals—and the reduction was greatest when small amounts of antigen were given. Taliaferro and Taliaferro (72) announced that splenectomy in rabbits injected with red cells significantly depresses antibody formation if performed during the four days after immunization. It is ineffective if done after the sixth day, indicating that most of the hemolysin production by the spleen is limited to a short period. Garvey, Dodd, Stahly, and Myers (73) find antibody in various tissues that contain reticulo-endothelial elements and also in the kidney cortex.

Rowley (74) compared the titer of hemolysins and hemagglutinins in normal human beings and in patients whose spleens had been removed from 8 days to 40 months previously. Small antigen injections were given, and the patients showed far less antibody than the controls. In splenectomized rats, Rowley finds (75) little or no antibody formation when small amounts of antigen are injected into the blood stream. By contrast, if antigen is injected intraperitoneally, intradermally, or intraportally, antibody formation is quite as good as in normal rats. He observes that the local distribution of antigen and the sites at which antibody is produced differ with the portal of entry of the antigen.

X-radiation of the Spleen. About 1905, while the efforts of the earlier workers to demonstrate antibody production by the spleen were still inconclusive, others studied the effects of splenic disorder induced by X-rays. Since most of this work involved simultaneous radiation of other lymphoid tissues the matter will be discussed in the following section. In a very recent paper, however, the function of the spleen itself has been brought out by Jacobson, Robson, and Marks (76), who irradiated splenectomized and normal rabbits both with and without lead shielding of the appendicular region or the exteriorized spleen. The capacity to form hemolysins following small intravenous injections of sheep cells was better by far when either the spleens or the appendicular regions were shielded. Moreover, when the shielded spleen or the shielded appendix was removed a few days after irradiation antibody formation still continued, showing that the intact lead-shielded spleen or appendix seemed to be able to initiate the production of antibodies or to make it possible for the process to be continued. More will be said of this below.

ANTIBODY FORMATION AND LYMPHOID TISSUE, LYMPHOCYTES, AND PLASMA CELLS

Lymphoid Tissue. It has long been known that lymph nodes take part in infectious processes. In 1890 Oertel (77), and in the next year Welch and Flexner (78), described lymphoid tissue changes in diphtheria, as did others too. Councilman (79) called attention to the collections of lymphocytes about inflamed areas, and the morphologists, particularly Flemming (80), Matko (81), Hellman (82, 83), and Ehrich (84, 85, 86) and others, drew attention to nodal changes.

About 1905 Helber and Linser (87, 88), Heineke (89), and Warthin (90) called attention to the effects of X-rays upon lymphoid tissue. In 1915 Hektoen (91, 92) X-rayed animals, reduced the lymphoid tissue, and found a lowered antibody response. He concluded that the spleen, lymphoid tissue, and bone marrow were the chief sites of antibody formation. Shortly thereafter Murphy and his collaborators, Sturm, Ellis, Taylor, and Witherbee, destroying the lymphoid tissue in animals by X-rays, stressed its importance and that of the spleen in antibody formation, and apparently ruled out the bone marrow (93–99). Shortly thereafter Murphy and Sturm (100) and Nakahara (101) went further, showing that dry heat stimulated lymphoid proliferation and increased antibody formation. Hussey (102) depleted the lymphoid tissue of guinea pigs by prolonged irradiation, and showed that as long as the depletion continued, anaphylactic shock was inhibited. This group of workers also demonstrated (97) the importance of the lymphocyte in the immunity of animals to transplanted tumors.

Bunting (103) suggested that macrophages merely destroy bacteria and that the lymphocyte is active in antibody formation.

Shortly thereafter some work from our laboratory, carried out with Hudack (104–106), indicated that lymph nodes nearest to the site of intradermal injections of antigen formed antibodies in high concentration at the same time that the merest traces appeared in the blood, and while there was no antibody elsewhere in the body. A series of experiments ruled out, in the following manner, the possibility that the antibodies found in the nodes had been formed elsewhere in the body. By injecting two different antigens, one in each ear of a large group of mice, the blood vessels of the ears on both sides and of the draining nodes on both sides were rendered more permeable than normal. Therefore these

tissues on both sides had equal opportunity to take out from the blood and to concentrate antibodies formed elsewhere in the body. But the agglutinin to the antigen injected in the right ear appeared first only in the lymph nodes draining that ear, and vice versa, while antibodies were not detectable in the ear tissue. Later, in experiments done with Kidd (107), lymph nodes nearest to the site of injection formed neutralizing substances first to vaccinia virus. The work was planned neither to show whether antibodies can be formed in other tissues nor to cast light upon the types of cells involved. The findings have since been confirmed with a variety of antigens by Burnet and Lush (108), Harris, Ehrich, White, and Dougherty, and others whose papers will be referred to below.

Lymphocytes. Soon after this, interest became focused upon the types of cells involved. Rich, Lewis, and Wintrobe (109) described the behavior of lymphoid splenic tumor cells in rabbits given sterile proteins, and they called attention to the possible relation of these lymphoid cells to immunity.

Ehrich (110) with Harris (111, 112, 113) together with Mertens and Grimm (114, 115) carried out an exhaustive study of the relationship of the lymphocyte to antibody formation. Injecting antigens into the abdominal cavity (115), they found practically no antibody in granulocytes and macrophages of the peritoneal exudates, but much in the peritoneal fluids. Antigen injected into the blood stream, followed later by nonspecific irritation of the peritoneum, gave similar results. Clearly the antibody of the peritoneal fluid was the result of secondary concentration in an inflamed area.

By contrast, when Ehrich and Harris (111, 112, 113, 115) injected various antigens into the pads of rabbits' hind feet—often using one antigen in the right foot and a different one in the left—much antibody was found in the draining popliteal nodes and insignificant amounts at the site of injection, although many macrophages and granulocytes were there (115). These workers were the first to correlate (111–112) the antibody titers of the popliteal node, the serum, the afferent and efferent lymph, and the cells of the efferent lymph, with cellular changes taking place in the node, at various intervals after injecting the antigens into the feet. A lymphocytic hyperplasia in the node was followed by a great increase in the cell count of the efferent lymph and a rise in its antibody titer. The antibody content of the cells of the lymph was higher than

that of the lymph itself, and it became greatest at the time of the greatest rate of increase of titer in the whole lymph, rather than at the time of the highest titer of the lymph fluid (114).

After two antigens had been injected, one in each hind foot, incubation of the cells from the efferent lymph of one side with lymph from the other showed that antibody passed from the cells to the lymph but not from the lymph to the cells. There was no take-up of antibody from the lymph.

In another paper Harris and Ehrich (113) found antigenic material, composed of cells, apparently made available to the lymph node by the production of a soluble substance of the same immunological specificity as the antigenic material found at the site of the injection. They suggested that phagocytosis and intracellular digestion of the antigenic particles, perhaps by macrophages, may have prepared the antigenic material for the lymphatic tissue.

Oakley, Warrack, and Batty (116) found secondary stimulation of horses, guinea pigs, and rabbits by subcutaneous injections of alum precipitated toxoid, capable of producing antitoxin in the draining lymph nodes. They did not intimate whether lymphocytes, plasma cells, or reticulo-endothelial cells were involved. Possibly, too, antibody was formed in the skin if the latter had been preinjected often enough to produce a granuloma. Again experimental findings point to the possible involvement of macrophages in antibody formation, but again, too, lymph node cells of some sort are certainly implicated. Indirect evidence for the role of lymphoid tissue has been presented by Stoerk and Eisen (117, 118), who report that pyridoxine is necessary for the maintenance of lymphoid tissue in rats. Pyridoxine deficiency cuts down antibody formation.

Miller and Favour (119, 120) obtained white blood cells from tuberculosis patients or guinea pigs sensitized with killed bacilli, and by special methods separated them into suspensions rich in lymphocytes alone and neutrophiles alone. Prolonged incubation of these cells, washed and resuspended in the plasma of unsensitized subjects, led to the development of a cytolytic factor in the plasma, the origin of which seems to be the lymphocyte.

Recently Kidd and his colleagues (121–124) have restudied the role of the lymphocyte in the regression of certain transplantable tumors. A few days after C_3H mammary carcinoma or lymphosarcoma cells are

implanted into racially resistant mice, the draining lymph nodes of the hosts show an increase in the number of germinal centers; cells, apparently plasma cells, proliferate in great numbers. When the growths regress the hyperplasia fades (121). The elimination of the transplanted tumor cells apparently takes place by the envelopment and destruction (122, 123) of the tumor cells by lymphoid cells. Minced lymph node material from mice that have previously overcome a transplanted lymphosarcoma, when incubated with the tumor cells, inhibits or abolishes the ability of the cells to proliferate when transplanted into susceptible hosts (124). These suggestive findings clearly emphasize the importance of lymphocytes or lymphoid cells in the processes involved in the immunity of the hosts to the tumor cells.

Depression of Antibody Formation by Lymphocytotoxic Substances. The evidence for the role of lymphoid tissue and the lymphocyte in the production and transport of antibodies has recently stimulated more workers to test the effects of lymphocytotoxic substances on antibody formation. Some of the work illustrating the effects of X-rays has already been mentioned, and more will be said about the matter below after considering some aspects of the influence of hormones on lymphoid tissue. For a full consideration of the effects of X-ray on antibody formation the reader is referred to a review, just published, by Taliaferro and Taliaferro (240) and also to an earlier one by Craddock and Lawrence (155).

Recently Spurr (145) found nitrogen mustard suppressing antibody formation. Nevertheless its lymphocytotoxic effect failed to produce the non-specific anamnestic response, about which more will be said below. He feels that the toxicity of nitrogen mustard interferes with antibody formation, and that his finding does not contest the role of the lymphocyte. More recently Bukantz, Dammin, Wilson, Johnson, and Alexander (146) have found nitrogen mustard suppressing antibody formation and cutaneous hypersensitivity in rabbits receiving horse serum. Schwab, Moll, Hall, Brean, Kirk, Hawn, and Janeway (147) have found both X-radiation and nitrogen mustard inhibiting the formation of precipitins in rabbits. The animals that showed little or no antibody formation also failed to develop the characteristic vascular lesions of the hypersensitive rabbit.

By contrast, Phillips, Hopkins, and Freeman claim that nitrogen mustard, having damaged the lymphopoietic tissue, failed to delay the anti-

body response to ricin, or to reduce the maximum serum antibody level (148).

The Relation of Pituitary Adrenocortical Function to Lymphoid Tissue, Lymphocytes, and Circulating Antibody. While the work of Ehrich and Harris was appearing, White and Dougherty and their colleagues studied the influence of pituitary-adrenal control over lymphoid structure and function. Stimulation of the pituitary, or injection of pituitary adrenotropic hormone, or adrenal cortical steroids oxygenated in position 11 of the steroid nucleus, led to a decrease in lymphoid tissue (125), as had been observed by others. A single injection of either type of hormone in mice was followed by an acute lymphopenia within a few hours (126). By contrast, the hormones had no effect upon blood lymphocytes in the absence of the adrenals (127, 128). An histological study (129) of lymphoid tissues, following antigen injections of these hormones, disclosed phenomena termed dissolution of the lymphocytes, that is, karyorrhexis, pycnosis, and a shedding or budding of the cytoplasm. Within lymph nodes there appeared, too, an increase of immature plasma cells (130) a few hours after the injections. Many small lymphocytes developed basophilia, suggesting an increase of cytoplasmic nucleoproteins and active protein synthesis. Injections into normal mice apparently increased β- and γ-globulins in the blood at the same time that dissolution of the lymphocytes occurred, suggesting that they may have contained the globulins and that the release—apparently under control of the pituitary-adrenal mechanism—presumably was effected through the shedding of cytoplasm.

At about the same time Kass (131) also reported the finding of γ-globulin in lymphocytes.

Dougherty, Chase, and White (132) found no antibody in extracts of lymphocytes from normal animals, but lymphocytes from immunes yielded much. Accordingly they (133) sought for evidence of the possible release of antibody globulin in a rise of the titer of blood antibody following injection of the hormones into hyperimmunized rabbits. Accompanying the resulting dissolution of lymphocytes, blood antibody rose slightly, and both phenomena were at their peak at the same time.

In further work the non-specific anamnestic response (134) was induced in previously immunized rabbits having at the time no demonstrable antibody in the blood. Injections of hormone into these animals

led to the appearance of antibody in the blood. Properly graded irradiations with X-ray also (135) brought on dissolution of lymphocytes, the anamnestic response and lymphopenia in normal animals, but failed in adrenalectomized ones. Much larger doses of X-ray precipitated the phenomenon even in the absence of the adrenals.

From this work the authors postulate an hormonal control over the level of circulating lymphocytes and the liberation of antibody through their dissolution. They have not claimed that lymphocytes form antibody but that they contain it, and that the primary site of antibody formation is still a matter of conjecture (136). However, since transplanted tumors, containing antibody-carrying lymphocytes (136, 137), seemed to have as much immune globulin as the original tumor tissue, these authors suggest that the globulin constituent of the cytoplasm apparently passes from cell to cell as the lymphocytes reproduce themselves. They have published several reviews of their own work (136, 138, 139).

Divergent Views. Having considered some of the evidence for the importance of lymphoid tissue in the processes of antibody formation, and for the part played by humoral control of circulating lymphocytes and antibody, we are now in a position to discuss a number of works which call into question one or the other, or both, of these mechanisms. To bring out the divergence of opinion more clearly some of the recent papers, that might have been mentioned earlier, will be discussed in this section.

Concerning hormonal control of antibody release or formation.—In 1947 Murphy and Sturm (140) concluded that cortical hormone is not essential for the release of antibody. Rabbits adrenalectomized by them developed an extreme hypertrophy of the lymphoid system. In the absence of the adrenals there could have been no effects either from the pituitary adrenotropic hormone or from cortical ones. Nevertheless the circulating lymphocytes increased and, if the animals were injected with antigenic materials, the lymphocytes increased enormously, a phenomenon not seen in normals. Rabbits given horse serum intraperitoneally and adrenalectomized ten days later produced excessively heavy precipitins. Cortical hormone given to adrenalectomized animals prevented the overdevelopment of the lymphoid system but did not reduce the increased antibody formation, probably, they believe, because of the disruptive effect of the hormone on the lymphocytes with more rapid

release of globulin. Eisen, Mayor, Moore, Tarr, and Stoerk (149), using quantitative methods for antibody determination, have reported that adrenalectomized rats, some repeatedly injected during immunization with adrenal cortical hormones, and others not receiving any, had identical concentrations of serum antibodies and γ-globulins. They concluded that adrenal cortical activity is not essential for the release of antibodies or γ-globulin. Fischel, LeMay, and Kabat (150), also using precise quantitative methods, failed to find an anamnestic rise in blood antibody following subcutaneous injections of ACTH or exposure to X-rays, although the circulating lymphocytes decreased. The absence of the anamnestic response following the administration of nitrogen mustard as found by Spurr (145) has already been mentioned. Herbert and deVries (151) diminished the number of circulating lymphocytes in human beings by injections of ACTH—most workers seem to be in agreement that this phenomenon occurs—but, in disagreement with Chase, Dougherty, and White, they found no concomitant rise of antibody. In contrast to this, Hammond and Novak (152) repeated the work of Dougherty, Chase, and White (134) exactly and, like them, obtained an anamnestic response. Nevertheless, when lymphocytes from immunized rabbits were injected into others, given in turn adrenal cortical steroids, no release of antibody occurred. However, these injected cells were foreign lymphocytes which had been washed and, as will be seen below, they may well have lost their antibody. Antopol (141) found excessive dosages of cortisone, given to normal animals, depressing antibody formation—apparently by bringing about the changes seen in the alarm reaction. According to Germuth and Ottinger (142) both compound E and ACTH inhibit experimental hypersensitivity to egg white in the rabbit. Both compounds, as shown by antibody N determinations, depress antibody formation. Compound E is much more active in both processes than ACTH. Both these hormones given to rabbits, by Björneboe, Fischel, and Stoerk (143), reduced the concentration of circulating antipneumococcal antibody and produced lymphoid tissue atrophy and a fall in the number of mononuclear cells. Harris and Harris (144) found cortisone exerting but little if any effect upon antibody production, although a drop in circulating lymphocytes and eosinophils occurred. The Arthus phenomenon and the state of anaphylaxis were not affected. However, space forbids a review of the

effects of these substances upon hypersensitivity, anaphylaxis, and the Arthus or Schwartzman phenomena.

Concerning the role of lymphoid tissue or lymphocytes in the processes of antibody formation.—Andreasen, Bing, Gottlieb, and Harböe (153) found no fall in plasma proteins in rats deprived of 90 percent of their lymphoid tissue. However, ablation experiments are difficult to interpret when the functional capacity of the remaining tissue is unknown. Habel, Endicott, Bell, and Spear injected anti-typhimurium rabbit serum intravenously into normal rabbits and animals previously inoculated with 1.0 ml. of P. tularense antigen (154). Other rabbits received two different antigens, in the foot pads of the opposite sides. They found the antibody titers of serum, lymph, lymph cells, and carefully washed cells from the nodes giving no clear evidence in favor of antibody formation by the nodes, or its transport in lymphocytes. They believe that the high antibody titer of lymph may be accounted for by seepage from the blood. However, these authors used very large injections of antigen which may have flooded the body and called out antibody production from many sites other than the draining nodes nearest to the site of injection. More will be said of this below.

Craddock and Lawrence (155) irradiated rabbits with moderate dosages of X-ray eight hours before immunization was begun. A severe lymphopenia developed and antibody formation was temporarily depressed. By contrast, irradiation given after antibody formation was under way but before it had reached its highest peak, failed to reduce the subsequent antibody titer of the blood. Although the blood lymphocytes fell, the dissolution of the lymphocytes apparently did not release antibody, nor did the damage done to the lymphoid tissue prevent further antibody formation. These authors suggest that the fall in antibody formation following Roentgen irradiation is not caused entirely by damage to the lymphoid tissue, and that, once antibody formation has been initiated, other more resistant tissues may assume this function. These authors, together with Valentine (156), have found no significant effect of adrenal cortical hormone upon the rate of production of thoracic duct lymphocytes in normal or adrenalectomized cats. Further (157), after giving typhoid vaccine subcutaneously and intraperitoneally to cats, they found no evidence of antibody in the thoracic duct lymphocytes and no antibody rise in the thoracic duct lymph after

X-radiation or administration of the hormone. A rise of antibody titer of the blood was seen in half of the animals. In evaluating these findings it is to be stressed that thoracic duct lymphocytes are cells which may have given up antibody before reaching the duct. More will be said of this below.

Technical Factors Giving Rise to Some of the Diverse Views. Reasons for the failure of some of these authors to find antibody associated with lymphoid tissue or lymphocytes have been given by Harris and Harris (158) in an excellent discussion of various biological and technical factors involved in the demonstration of antibody in these structures. Calling to mind the fragility of lymphocytes (114) they demonstrated, in new experiments, the loss of antibody from these cells during laboratory manipulations such as washing. They stressed the particular importance of the route of entry taken by the antigen and the confusing effects of injecting large doses. For example, some rabbits were given only 0.2 ml. of a dysentery antigen suspension in one foot while others received five times as much. The draining, popliteal nodes of the injected sides showed relatively more antibody when the smaller dose was used. Evidently the larger doses flooded the body, and the spleens and the nodes from the uninjected sides showed more antibody. A second antigen injected subcutaneously into the backs of some of these animals—thereby reaching the popliteal nodes of both sides equally—called out equal antibody formation on both sides; an experiment offering clear-cut evidence of antibody formation in the nodes draining the injected legs, and not the seepage of antibody from the blood.

Further, the Harrises (159) have lately injected serologically distinct strains of influenza virus separately into the right and left hind-foot pads of rabbits. Antibodies to each strain appeared earlier in the respective local lymphatic systems than in the blood, and antibody to the antigen injected on the right side appeared in much higher concentration in the right popliteal node than in the left one, and vice versa. In these experiments the appearance of antibodies coincided with a cortical lymphocytic hyperplasia in the nodes. The ratio of antibody in the cells of the efferent lymph to that of the lymph itself ran as high as 16:1, and was highest at the time when the rate of increase of antibody in the local lymphatic system was also greatest. By the fifth day following the injections the serum antibody titers to both virus strains exceeded the lymph node titers on both sides. In spite of this the nodes of each side showed

only low antibody titers to the antigen injected on the opposite side. Even if one assumes that the low antibody titer to antigen injected on the opposite side was due only to seepage from the blood, which is doubtful, surely the far higher titer of antibody to antigen injected on the same side cannot be accounted for in this way.

Antibody Formation and Plasma Cells. In 1913 Huebschmann (160) observed collections of plasma cells in the spleen during the course of infectious conditions, and he related the phenomenon to antibody formation. Since then many other clinicians or pathologists, among the earliest Perlzweig, Delrue, and Geschickter (161), Osgood and Hunter (162), Markoff (163), and Bing and Plum (164), have associated high blood globulin levels with plasmacytosis and antibody formation in, for example, multiple myeloma, plasma cell leukemia, serum sickness, and agranulocytosis, respectively. The latter authors (164), among others, brought out the important fact that patients with plasma cell myeloma show high blood globulin levels, but patients with lymphatic leukemia exhibit no increase. In 1940 Bing (165, 166) suggested that the globulin of the blood is derived from plasma cells of reticulo-endothelial origin. In the laboratory, Downey (167) observed plasmacytosis in the marrow of a rabbit with a large abscess. Doan, Sabin, and Forkner (168), and Miller (169) found collections of plasma cells in the omentum of rabbits injected with tubercle proteins. The splenic lymphoid tumor cells of Rich (109), already referred to, and believed by him to be related to immunity, are considered by some to be plasma cells.

In 1938 Koluch (170) described the development of bone marrow plasma cells from reticular plasma cells in rabbits that were sensitized and then shocked, and he suggested a direct relationship between this transformation and antibody formation. Björneboe and Gormsen (171, 172), with Lundquist (173), hyperimmunized animals with multiple injections of various antigens, and found collections of plasma cells in the spleen, liver, fatty tissues of the renal pelvis and in the medullary cords of the lymph nodes. An accompanying increase in the blood globulin was attributed wholly to antibodies. Later (174) Koluch, Good, and Campbell extended the previous observations of the former. They observed an increase in a specific type of bone marrow reticulum cell following sensitization of rabbits. Subsequent anaphylactic shock initiated a cycle by which these reticulum plasma cells became Marshalko type plasma cells. Further, a patient with serum sickness showed reticular

plasma cells and various other stages of developing plasma cells freely circulating in the blood. In the past year Good and Campbell (175) found, in patients with acute rheumatic fever and streptococcic pharyngitis, a plasmacytosis of the bone marrow associated with increased serum globulin, while in states of inactive rheumatic fever the number of marrow plasmacytes and the serum globulin lay within normal ranges. Since Anderson, Kunkel, and McCarty (176) find variations in the serum γ-globulins reflecting antibody formation against group A streptococci, Good and Campbell suggest that marrow plasmacytosis is correlated with antibody formation and that it is the morphological expression of the response of the reticulo-endothelial tissues (175) to the antigen of the streptococcus. Further work (177) including studies on allergic encephalitis (178–181) suggests to them that the local secondary response in an animal that has had previous contact with an antigen is an accumulation of plasmacytes in the exudate.

Is it not probable that the time has come to call for more precise differentiation of cell types? The cells appearing in inflammatory exudates are called macrophages by some observers, lymphoid by some, and plasmacytes by others. This difficulty will be illustrated again below. In another paper Campbell and Good (181) have suggested the development of plasma cells from invasive lymphocytes, from reticulum cells of the pia arachnoid, from macrophages, or even from Kupffer cells in the liver—in short from various multipotent connective tissue cells—and they feel that after reaching maturity the plasma cells degenerate and disappear. By contrast, Bueno (182) believes that plasma cells may not be doomed to degeneration; they may be cells in a certain stage of the resistance process which can, he believes, change back into reticulum cells in the lymph nodes, at least in guinea pigs during prolonged vitamin C deficiency, and perhaps in chronic inflammatory conditions in man.

Surely the time has not yet come to settle these differences of opinion and only the expert cytologist of the future will be qualified to attempt the task.

In the meantime, Astrid Fagraeus (183, 184, 45) reports a change of reticulum cells to plasma cells in the red pulp of rabbit spleens two or three days after intravenous injections of various antigens. Bits of red pulp (mostly plasma cells) and bits of follicular tissue (chiefly lymphocytes) were taken from these spleens and grown in tissue culture

for short periods of time. The bits of red pulp produced much antibody, in proportion to the concentration of the plasma cells. The follicular, lymphocytic bits, produced little antibody. The difference was greatest four days after a secondary antigen injection and—it should be stressed—in those pieces with immature plasma cells. Fagraeus concluded that the lymphocyte seems to have little to do with antibody formation and that reticulo-endothelial elements produce antibodies by developing into a type of cell with the morphological characteristics of plasma cells.

So does contemporary thinking swing back to earlier reticulo-endothelial hypotheses of antibody production.

The work of Fragraeus emphasizes splenic tissue function. The plasma cells of lymph nodes, too, have come in for study. Ehrich, Drabkin, and Forman (185) injected various antigens into the foot pads of rabbits and studied the draining lymph nodes histologically by the methyl-green pyronine technique for evidence of increased protein synthesis—that is, for ribonucleic and desoxyribonucleic acids. The findings were correlated with chemical estimations of these substances in the node tissue and serological studies for antibody content. Following the injections of antigen the ribonucleic acid increased and reached its peak by the fourth to sixth days, at the same time that antibody formation also reached its peak. During the first six days the cellular reaction was chiefly that of plasma cells, first plasmablasts then mature plasma cells with ribonucleic acid contained in them. The change occurred mostly in the medullary portion of the node, not in the cortex. Lymphocytes showed their greatest activity after the antibody peak was over. These authors interpret their findings as indicating that the plasma cell and not the lymphocyte is responsible for antibody formation.

Even more lately Reiss, Mertens, and Ehrich (186, 187) have observed certain lymphoid cells *in vitro* following their collection from popliteal efferent lymph of rabbits injected into the foot pads with typhoid H or brucella antigens. These cells, which agglutinated on their surfaces the bacteria to which the animals had been immunized, were identified as belonging to the plasma cell series. Lymphocytes failed to show the phenomenon. The authors feel that the plasma cell rather than the lymphocyte elaborates agglutinins.

Ehrich (185) has commented on his earlier work in which much more antibody was reported in lymphocytes of efferent popliteal lymph than in the lymph itself—following antigen injections into the feet of rabbits.

He now prefers the term lymphoid for these cells, including plasma cells, which also leave antibody-forming nodes with lymphocytes. He suggests that in the earlier work centrifugation of lymph, for antibody titration, may not only have spun down plasma cells with the lymphocytes but also large shed fragments of antibody-containing cytoplasm from plasma cells. In this way plasma cell antibody was mistaken for lymphocyte antibody.

Ehrich considers mature plasma cells the source of antibody, instead of immature ones as believed by Fagraeus—this for reasons too detailed (185) to include here. He pictures antibody synthesis in the cytoplasm of plasma cells, like hemoglobin synthesis in erythrocytes, accomplished during the maturation of these cells and completed when maturity is reached; but the maximal concentration of antibody is not reached until these cells become mature.

Despite all this, the case for the plasma cell is not yet complete. At the same time that Ehrich, Drabkin, and Forman were performing the experiments just reviewed, Harris and Harris (188) independently attacked the same problem with similar experiments and similar techniques and came to a different conclusion. The papers of both groups appeared in the same issue of the same journal (185, 188). The experiments of Harris and Harris with the methyl-green pyronine technique showed ribonucleic acid appearing early, on the second day after injecting antigen into the foot pads of rabbits, instead of on the fourth to sixth days, as in the experiments of Ehrich and his co-workers. The ribonucleic acid reaction was seen first in reticulum cells of the draining nodes, then in transitional cells, then in young, and finally in mature lymphocytes. The ribonucleic acid increased during the second to fifth days, and the peak of formation occurred just before the peak of antibody formation. Plasma cells there were in the medulla but most of the cellular changes and cells containing the specific staining lay in the cortical portion of the node where there was a diffuse lymphocytic hyperplasia. The authors consider the lymphocytes as the active cells.

Further, in one of the most recent papers by Harris and Harris (159) already discussed, it is to be recalled that different strains of influenza virus, injected into the feet of rabbits, called out in the draining popliteal nodes, coincidently with the appearance of antibodies, a cortical lymphocytic hyperplasia, not a plasmacytic one.

Clearly the disagreement in the interpretations of the findings in the

studies of these two groups of workers wait upon the future for an explanation. In the meantime would it not be well to consider both types of cells implicated, at least in the transport of antibody if not in its actual formation?

Perhaps studies of the sort now being carried on by Chase and others (189–205) should aid in determining the types of cells involved in antibody formation and transport. Chase (189) has presented clear evidence to show that guinea pigs sensitized to certain simple chemical compounds develop circulating antibodies to them in the serum. Skin sites of normal guinea pigs, prepared as for a Prausnitz-Küstner test with these sera, react when the animals are injected with the simple chemical itself. Further, the sera of animals sensitized to simple chemical compounds confer passive anaphylaxis. Gell, Harrington, and Rivers (190) have noted that rabbits, too, develop antibodies in their sera when injected with a variety of compounds directly reactive with proteins.

In earlier work (191) Chase produced local skin reactivity by the passive transfer of antiprotein sera. Chase and Landsteiner (192) found that white cells, from sensitized donor animals, were capable of transferring to normal animals a sensitization of the delayed contact dermatitis type, demonstrable when the recipients were tested with the sensitizing agent. Now Chase finds (189, 191–197, 199) cells of inflammatory peritoneal exudates, buffy coats, spleens and lymph nodes of guinea pigs, sensitized to picryl chloride or to tuberculin, capable of transferring sensitivity to both the delayed and the immediate types of skin reactions. Heating or freezing these cells destroys this capability. The cells are therefore not mere carriers of antibody; they have something that serves in the elaboration of antibody.

When many more cells than are necessary to produce skin sensitivity are taken from the spleens or lymph nodes of animals sensitive to picryl chloride and transferred to normal recipients, the uteri of these animals show anaphylactic contractions. These can be elicited by the Schultz-Dale technique, as early as the second or third day after the transfer, when the organ is challenged with guinea pig serum coupled to picryl chloride, as antigen. At the same time antibodies appear in the sera of the recipient animals. Freezing the cells before transfer destroys their capacity to produce dermal hypersensitivity and contractibility of the uterus. Extraction of the cells fails to disclose the presence of antibody.

Recently Harris and Harris (198) and Chase (199) have found that

a similar mechanism operates in the rabbit when common antigens are used instead of drugs. The other details outlined above have been confirmed.

The fact that transferred cells apparently yield something after transfer that cannot be recognized within the cells themselves, is further evidence for a significant role of lymphoid tissue in antibody formation.

The ability of cells to transfer tuberculin sensitivity to normal animals has been confirmed by Cummings, Hoyt, and Gottshall (200), Stavitsky (201), and Kirchheimer and Weiser (202). Metaxas and Metaxas-Bühler (203) have obtained the transfer of local cutaneous hypersensitivity to tuberculin in guinea pigs. In man the intradermal injection of leucocytes from tuberculin-positive patients to tuberculin-negative ones, by Lawrence (204), has led to the transfer of local cutaneous hypersensitivity to tuberculin. Local passive transfer of the immediate whealing type of cutaneous reaction has been accomplished by Waltzer and Glazer (205), who injected viable leucocytes from the blood of atopic skin sensitive patients into the skin of normal subjects. The transfer of red blood cells yielded negative results.

DOES ANTIBODY FORMATION TAKE PLACE WITHIN THE CENTRAL NERVOUS SYSTEM?

So much has been said about lymphocytes, plasma cells, lymphoid tissue, and the spleen in their relation to antibody formation that one may fail to recall that the latter two tissues are in considerable part reticulo-endothelial structures, active, as will be seen below, in the capture of antigen. Throughout this chapter the activities of reticulo-endothelial cells and the reticulo-endothelial origins of macrophages, plasma cells, and lymphocytes have been stressed.

Having discussed the evidence for the transport, as well as for the formation of antibody, by these types of cells, we find ourselves in a more favorable position to consider the reports of workers who have found high titers of antibody in the central nervous system of animals inoculated with a variety of neurotropic viruses (206–209). Whether antibody merely accumulates in the nervous tissue, by seepage from the blood following its production elsewhere in the body, or is formed there by reticulo-endothelial elements, such as microglia cells, astrocytes, or other cells, or whether antibody appears there because of the infiltration

of antibody-carrying lymphocytes or plasma cells has been under debate for some time.

Only a few recent contributions to this debate will be mentioned here. In 1925 Katzenelbogen (210) inclined to the belief that antibodies were formed within the central nervous system. The electrophoretic studies upon cerebrospinal fluid by Kabat, Moore, and Landow (211) demonstrated the formation of some γ-globulin by these tissues probably—as they suggested—by reticulo-endothelial elements.

Morgan (212) injected monkeys intracerebrally with poliomyelitis virus and demonstrated the highest antibody titers in the body within the anterior horns of the spinal cord and little or no antibody in the blood—a finding suggestive of antibody formation in the central nervous system itself, probably by reticulo-endothelial cells or by lymphocytes, plasma cells, or other cells, which accumulated in the tissues.

Olitsky, Schlesinger, and Morgan (213–215), studying the mechanisms of immunity in rabbits or guinea pigs to Eastern equine encephalomyelitis virus (EEE), or the Western type (WEE), found the serum: brain: spinal fluid ratio for antibody titers (214) to be about 300:3:1. However the ratio was not fixed and in occasional instances the brain titer increased so much that the serum:brain ratio reached 1:1 (216). The findings in this paper, and in another by the same authors (217), indicate that the amount of antibody in the serum is not the important factor in determining the immunity of a vaccinated animal, but instead the availability of antibody at the portal of entry of the virus, or in the tissues which are the main seat of attack by the virus. In later work, Schlesinger (218, 219) demonstrated the presence of a factor in the central nervous system of vaccinated mice which arrested multiplication of a slowly growing strain of WEE virus. This seemed to be a neutralizing antibody produced locally. Mice vaccinated but not challenged with virus showed very low serum titers. When challenged on the fifth day following vaccination the brain titer increased out of proportion to the blood titer. In another experiment, mice vaccinated with a mixture of inactive WEE and EEE viruses, in equal parts, were challenged intracerebrally with virus of either type, but not with both. In the brain tissue the concentration of antibody relative to the challenging virus increased, but antibody to the other type of virus did not increase. This finding cannot be explained by assuming a local accumulation of antibody, and it seems probable, if not certain, that antibody was formed locally.

Sabin and Steigman (220) obtained suspensions of anterior horn material from poliomyelitic monkeys about one month after paralysis had occurred, and found no antiviral activity in them. They concluded that their work does not support the hypothesis of local antibody formation in the nervous system, and that their findings are in accord with earlier work of Sabin and Olitsky (221).

One may well ask, in the present state of doubt—as suggested by Morgan (212) herself—whether regions of high concentration of antibody in the nervous system represent local formation or accumulation of antibody. The mechanisms for both are present since there is both an accumulation of lymphocytes and other cells of the monocytic series in the inflamed areas, and the presence of fixed microglia cells.

TRACER ANTIGENS AND THE SITES OF ANTIBODY FORMATION

The use of tracer antigens promises to yield knowledge about the sites at which antibody formation is initiated. Since other chapters in this volume will discuss this field of work, a review here would be out of place. Instead, mention may be made of findings from our laboratory which will not be presented later in this book. Certain azoproteins (222–224), formed from exceedingly diffusible blue dyes, have been shown to serve well as tracer antigens. Injected intravenously into mice these compounds are taken up predominantly by reticulo-endothelial cells throughout the body. In this respect our findings are in agreement with those of others (11, 225–238). It has recently been shown that two of these azoproteins, as also the plain proteins from which they are derived (223, 224), once taken up by reticulo-endothelial cells of the liver or mesenteric lymph nodes, may persist within these cells, retaining their antigenicity far longer than has generally been supposed. The material gradually and slowly disappears. By this process, antigen persisting in reticulo-endothelial cells, and slowly liberated, may serve as a simple stimulus for prolonged antibody formation.

Suffice it to say here that among the reticular and endothelial cells that take up and hold antigen—in lymph nodes, spleens, or elsewhere—we find the very ones which give rise to cells of the lymphoid and plasma cell series. How these daughter cells acquire their antibody is yet to be learned.

COMMENT

The attempt has been made, as set forth at the beginning of this chapter, to review impartially some of the trends of contemporary thinking about the sites of antibody formation, but without assuming, on our part, the task of serving as an arbitrator or critic—a task impossible in our present state of ignorance. Since the whole chapter is only a summary, no further condensation will be made.

Since lymphocytes and plasma cells are not phagocytic, it has been suggested by many, as already mentioned, that granulocytes, macrophages, or other reticulo-endothelial cells may prepare antigen in some way for the antibody forming cells, or stimulate them, whichever they may be, in some manner. Cells which develop as fast as lymphocytes or plasma cells must be peculiarly susceptible to modifying influences, as suggested by Burnet and Fenner (239). For a review of this subject, including various hypotheses for the mechanisms of antibody formation within cells, the reader is referred to the excellent monograph by these authors (239).

The sites of actual antibody formation still remain unknown, although much circumstantial evidence is at hand. We know something about the situations in which antibody is found and of the cells that apparently carry it. The finding of tracer antigens in reticular cells by most of the workers who have studied the subject (222–238) indicates, at least, some of the sites from which antigenic stimuli to antibody formation may be expected to take their origin. Besides this, the presence of antigen has been reported in many other types of cells, especially in the kidneys (222, 227, 228, 231, 234–238), and by Coons and Kaplan (234) in lymphocytes themselves. The latter authors found the pneumococcus polysaccharide persisting, as antigen, for long periods (234), but in a recent paper with Leduc (238) they find other, protein antigens persisting for only a few days. The reports of others (222–224, 227, 228, 235, 236) indicate that protein antigens may persist much longer than has generally been supposed within some of the reticular cells that give rise to daughter cells of the lymphoid and plasmacytic series. If these findings are correct, the problem may be greatly simplified for future attack. However, for the purposes of a review of this sort, the time has not yet come to speculate with profit upon what happens, in these cells or others, between the capture of antigen and the appearance of antibody.

At present the literature displays an extraordinary narrowness of mind, since macrophages, reticulo-endothelial cells, lymphoid cells, lymphocytes, or plasma cells are hailed by their proponents as though each was alone concerned in antibody formation. The presence of antibodies in the spleen, lymph nodes, and other tissues in which there is a great variety of cells still leaves us without proof that any one cell alone is concerned.

Present-day opinions on the subject of whether or not reticulo-endothelial cells form antibody seem to depend on the school of hematological cytology that one favors. But, until the cytologists can convincingly demonstrate just which cells are plasma cells and which are not, which are lymphoid but not lymphocytes, what the life cycle of the lymphocyte really is, and what is the relationship of these cells to one another and to macrophages, polymorphonuclears, and fixed reticulo-endothelial cells, we shall remain confused.

Until more light is shed upon this tortured subject it would seem best to maintain an open mind. Clearly various types of cells and various tissues are implicated. In the meantime, in order to evaluate the work of the past and to plan for that of the future, we must be constantly aware of the all-important influences of technical factors upon the conclusions that will be arrived at; that is to say, one must constantly consider the route by which antigens enter the body, the dosage of antigen, and the frequency with which the tissues are challenged.

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Chapter 3

THE RESPONSE OF IMMUNIZED ANIMALS TO SPECIFIC AND NON-SPECIFIC STIMULI

By Jules Freund, *The Public Health Research Institute of the City of New York*

When an animal receives an antigenic stimulus for the first time, antibodies will be demonstrable in the bloodstream after a period of lag, followed by a sharp rise, then a slower fall in titer. When the same antigenic stimulus is received a second time there is a more vigorous response: the rise in titer is faster, the peak is higher, and the fall is slower. The difference in the response of the animal to the primary and secondary stimuli varies greatly with different antigens.

The secondary response is of importance in prophylactic immunization of man and lower animals, in infectious diseases following immunization or following natural contact with the agent of infection, and also in hyperimmunization of animals. Circulating antibody titers following active immunization may not measure completely the acquired, specific immunity against an infectious disease. The specific resistance acquired by active immunization is often due to the combination of the antibodies present before infection and the secondary response that may occur as the result of infection. Therefore, a sharp distinction may be made in correlating antibody titers with protection between antibody titers following active and passive immunization.

The following experiment demonstrates the role of the secondary response that takes place after infection in immunized animals. Schlesinger (1) immunized mice with inactivated Western equine encephalitis virus and tested their resistance by intracerebral injection of large doses of virus. For the challenge, two variants of the same strain of virus were used. These variants differed in their rate of multiplication. The mice succumbed to the infection with the variant which multiplied rapidly but resisted the infection with the slowly multiplying strain. The latter was multiplying in the brain to reach a 100-fold increase in titer. Four days after challenge the brain became free of demonstrable virus. The correlation between the fate of the virus and the neutralizing anti-

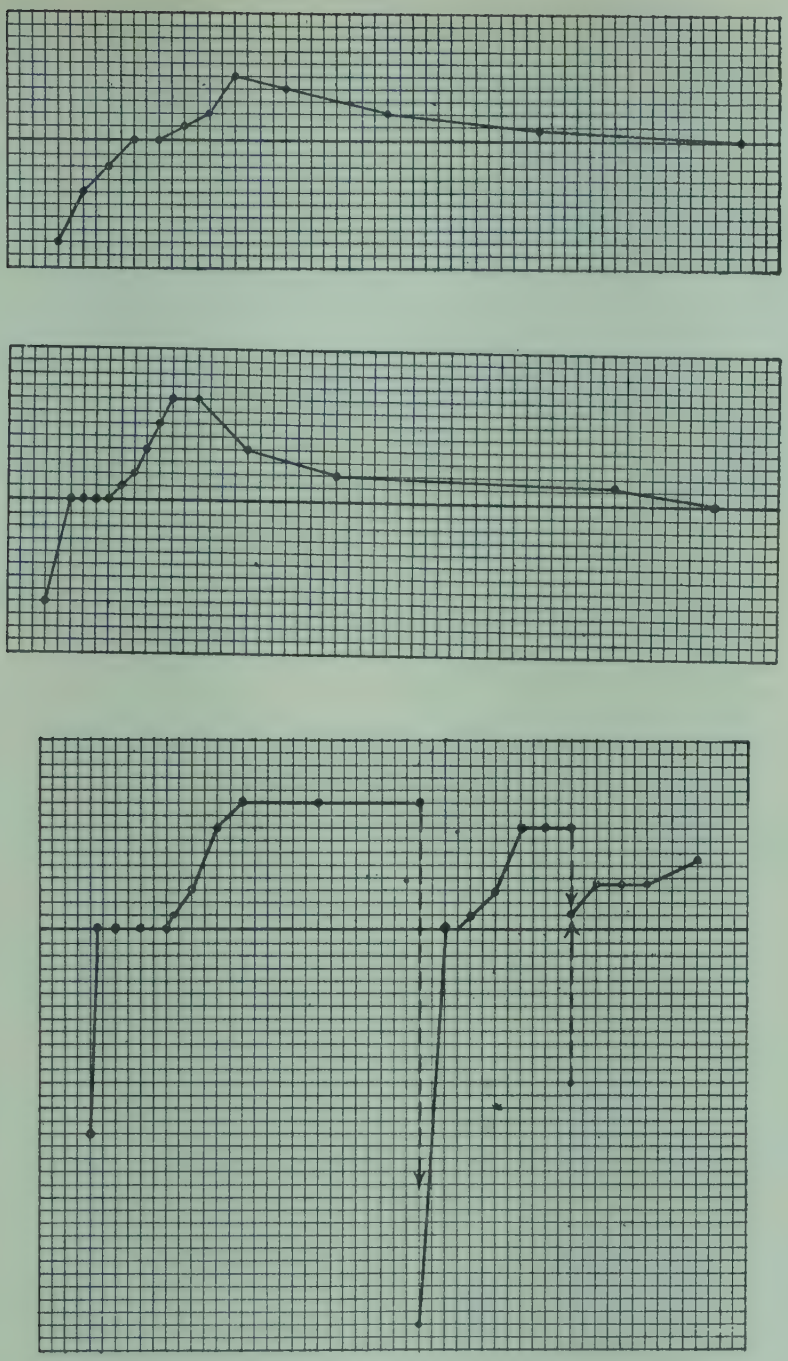

FIGURE 1. PRECIPITIN FORMATION AGAINST MAJA-PLASMA IN THE RABBIT; THE RATE OF DISAPPEARANCE OF THE INTRAVENOUSLY INJECTED ANTIGEN FROM THE BLOOD OF THE RABBIT

(From E. von Dungern, *Die Antikorper,* Jena, 1903)

Ordinate: Above heavy line, precipitin titer; below heavy line, estimated amount of antigen in the blood of the rabbit.

Abscissa: Time after injection, 12 hours per division.

Intravenous injections: First on Jan. 8, second on Feb. 7, third on March 6, fourth on March 20, fifth on March 26.

body titer of the brain (extract) was illuminating. Following challenge there was local antibody formation in the brain, and the disappearance of the virus from the brain four days after challenge was coincident with the appearance of neutralizing antibodies which had not been demonstrable in the CNS before challenge.

The nature of the response to the secondary stimulus was recognized with reference to diphtheria toxin by Solomonsen and Madsen (2) more than fifty years ago. Their observations, however, were not as clear-cut as those of von Dungern (3), who used identical amounts of antigen

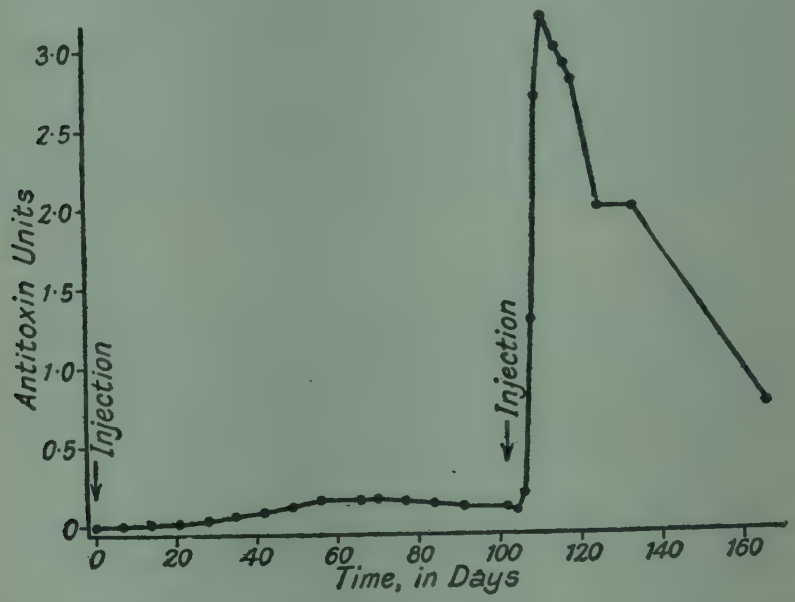

FIGURE 2. ANTITOXIN PRODUCTION IN THE HORSE, SHOWING DIFFERENCE BETWEEN PRIMARY AND SECONDARY RESPONSE
(After Glenny and Südmersen, *Hyg.* 1921, 20:176.)

for all injections. Von Dungern injected rabbits intravenously with *maja*-plasma and measured antibody formation by precipitin titrations. *Maja* is a crab whose plasma is supposed to have a single antigenic protein. The main points von Dungern established were: the lag period before antibodies are demonstrable in the blood is shorter after the secondary than after the primary stimulus; the rate of antibody formation and the peak are higher and the duration of antibody formation longer after secondary or tertiary stimuli. He also found that the response of the animal is about the same regardless of whether the secondary stimulus is applied when the antibodies are still present or after

they can no longer be demonstrated in the blood. These observations were published in 1903 and little has been added to them that could be called illuminating.

The difference in the responses to the primary and secondary stimuli is most obvious when antigens are used which induce a particularly slow response. Of the exotoxins, diphtheria toxin is a good example. Glenny and his associates (4) published many observations with reference to diphtheria antitoxin formation in man and lower animals. Since in most of these experiments not toxin but a mixture of toxin and antitoxin was used and the animals were injected subcutaneously, attention may be called to Burnet and Freeman's results (5). They injected staphylococ-

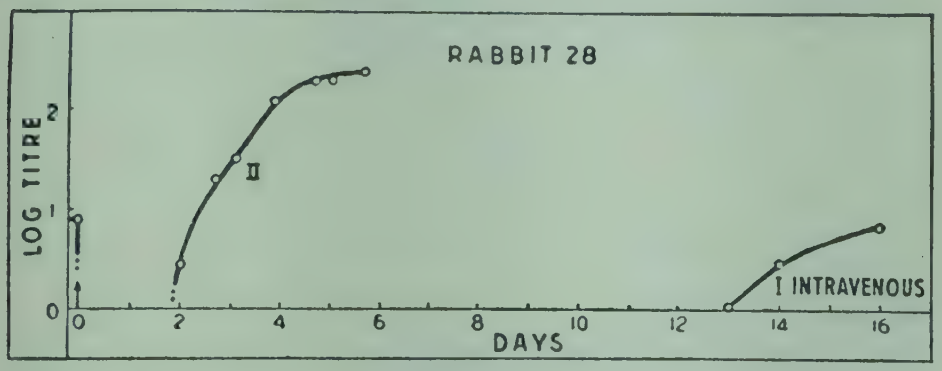

FIGURE 3. ANTITOXIN FORMATION TO STAPHYLOCOCCUS TOXIN
I, first injection; II, second injection
(After Burnet and Freeman, *Path. Bact.*, 1932, 35:477.)

cus toxoid directly into the bloodstream of rabbits and found circulating antitoxin after a lag of 13 days following the first injection and a sharp rise of titers three days after the second injection. The difference between the primary and secondary responses is very sharp when rabbits are immunized with killed typhoid bacilli; as this is seen in the graphs presented by Topley, Wilson, and Miles in their well-known book (6).

Secondary response may fail to occur with some antigens. Burnet and Freeman (7) obtained no secondary response in rabbits after intravenous injections of rickettsial (of Q fever) suspensions. In this case the explanation may lie in the intracellular persistence of the parasitic antigen till the second injection.

Immunization of man with the specific carbohydrates of pneumococci from the standpoint of secondary response merits special consideration. Heidelberger, MacLeod, Kaiser, and Robinson (8) immunized a large

number of medical students against pneumococci and measured antibody formation by fairly frequent antibody nitrogen determinations. Many samples of blood were also tested by mouse protection tests. Minute amounts of antigen in salt solution, either killed pneumococci or purified carbohydrate, were injected intracutaneously or subcutaneously twice within one week. The resultant antibody formation lasted for more than two years. The antibody level was almost constant during the first five to eight months and then it began to fall gradually to a half

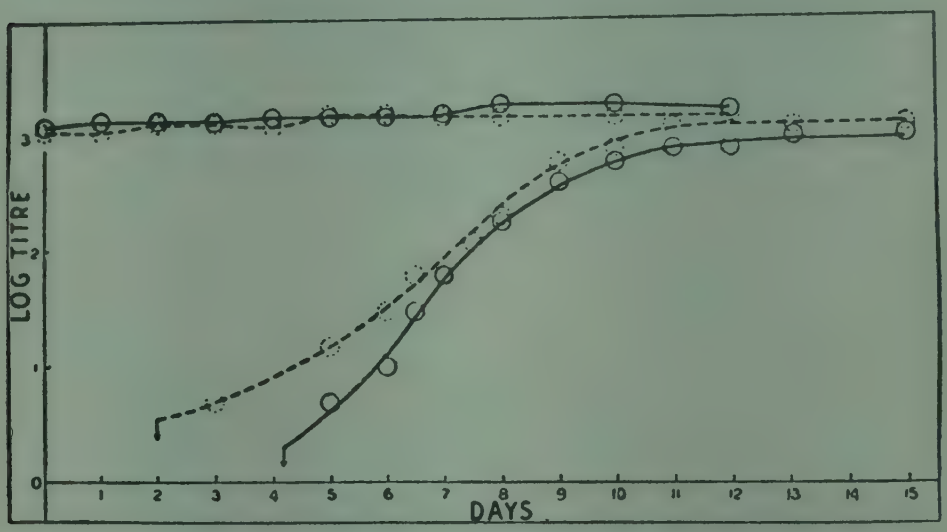

FIGURE 4. PRIMARY AND SECONDARY RESPONSE OF TWO RABBITS GIVEN RICKETTSIAL EMULSION INTRAVENOUSLY
There is practically no secondary response in either animal. (From *Med. J. Australia,* 1938, 1:296; also printed in Burnet and Fenner, *The Production of Antibodies,* 2d ed., Melbourne, 1949.)

to one tenth of the peak level. After two years, antigen was again injected but no rise in the antibody level was found; thus, a secondary response to this antigen was not detected. The explanation of the failure to respond to such widely spaced injections of antigen may be, as the authors suggest, that unlike proteinic antigens, the specific carbohydrate is resistant to the enzymes of the host and may persist in the antibody-producing cells for a long time. Indeed, Felton (9) recovered specific carbohydrates of pneumococci from various organs of mice injected with Type I and II carbohydrates as much as a year earlier. The relationship of dosage to antibody formation with reference to the specific carbohydrate of the pneumococcus is probably related to the problem of

secondary response. Schiemann and Casper (10) discovered that mice can be immunized with purified specific carbohydrate of pneumococcus Type I, but immunity failed to appear if the dosage was large. Moreover, Felton (9) found that mice that had received a large dose of purified antigen, in excess of the amount optimal for immunization, could not be protected by subsequent injection of 0.0005 mg. antigen. Immunity was measured by graded doses of pneumococci injected into the peritoneal cavity of mice. The "paralyzing" activity of a large dose of antigen was found to be type specific and lasting for more than one year. As mentioned above, type-specific carbohydrate can be recovered from the organs of these mice for several months after injection. There is no statement in Felton's publications with reference to the question as to whether passively introduced antibodies against the specific carbohydrate remain demonstrable in the blood of "paralyzed" mice bearing antigen in their organs. It is possible that passively introduced antibodies would combine with the excess antigen in the tissues of the mouse and would not be found in the blood stream.

The most clear-cut demonstration of the secondary specific response is immunization by two single administrations of antigen separated by long intervals of time. The secondary response, however, is well manifested when two widely separated series of injections—for example, of diphtheria-toxoid—are given (11). The second series of injections at first produces an impressive rise in antibody formation, but in spite of repeated antigenic stimuli the titer decreases. After a period of rest a third series of injections of alum toxoid produces a moderate rise in antibody titer.

It is usually said that after primary stimulus antibodies appear after a considerable lag and the rate of their formation is low. This is true for diphtheria and tetanus antitoxins but does not hold for some other antibodies. After the intravenous injection of formalin-killed typhoid bacilli, H agglutinins are often detectable in low titers, 1:5, 1:10 in two to three days, reaching a titer of 1:300 in about ten days. This can be particularly well seen when the test is read after centrifugation. Rabbits injected with killed pneumococci were found by Curnen and MacLeod (12) to be protected against infection within two days after injection. Circulating antibodies were demonstrable four days following a single injection of killed pneumococci. Spectacular observations were reported by Nunes (13) from E. G. D. Murray's laboratory. Guinea pigs were injected intra-

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peritoneally with an 18-hour culture of pneumococci Type I combined with carmine. Carmine blocks the phagocytosis of pneumococci and thus promotes infection. Blood samples were taken five and sixteen hours after injection. Five hours after infection agglutinins were found in 7 of 18 animals and sixteen hours after infection in 5 of 25 guinea pigs. The titers ranged from 1:4 to 1:256. Two more groups of animals were injected with pneumococci combined with carmine and given sulphamethazine immediately after infection. The sera of these animals had no demonstrable agglutinins during the first sixteen hours after infection. No agglutinins were found in the sera of experimental guinea pigs before infection. This report suggests that at least under the conditions of this experiment antibody formation may occur within a few hours after a primary infection. The experiment is not convincing unless it is supported by a specific antibody absorption test and unless it is shown that the agglutinin containing sera protect mice against infection with pneumococci Type I. Another possible reservation against the experiment might be the assumption that animals producing antibodies so promptly had been stimulated before, perhaps by some antigen related to the pneumococci, and had no detectable antibodies before infection. Nunes's observation, however, is in line with the experiments of Schoenheimer, Ratner, Rittenberg, and Heidelberger (14) showing that isotopic tyrosine can appear in the antibody 5.5 hours after feeding this amino acid to a rabbit producing antibodies against Type III pneumococci. There is a difference between the two experiments which may separate them. Nunes reported that pneumococci induced the appearance of circulating antibodies in five hours, while the experiments with isotopic tyrosine showed that, in the rabbit that had been producing antibodies, synthesis of or at least changes in the polypeptide-bonds of the antibody molecule can occur in 5.5 hours.

When about 10,000 killed typhoid bacilli are injected intravenously into rabbits circulating agglutinins usually fail to appear. When a few weeks later, the rabbits receive similar injections, the responses are of the secondary type. This observation might suggest the possibility that a single injection of fluid diphtheria toxoid resulting in circulating antitoxin production only after about three weeks may serve both as a primary and secondary stimulus. It is conceivable that the appearance of antitoxin is the result of two phases of antigenic stimulation. The first of these may be undetectable and the second occurs when the persisting

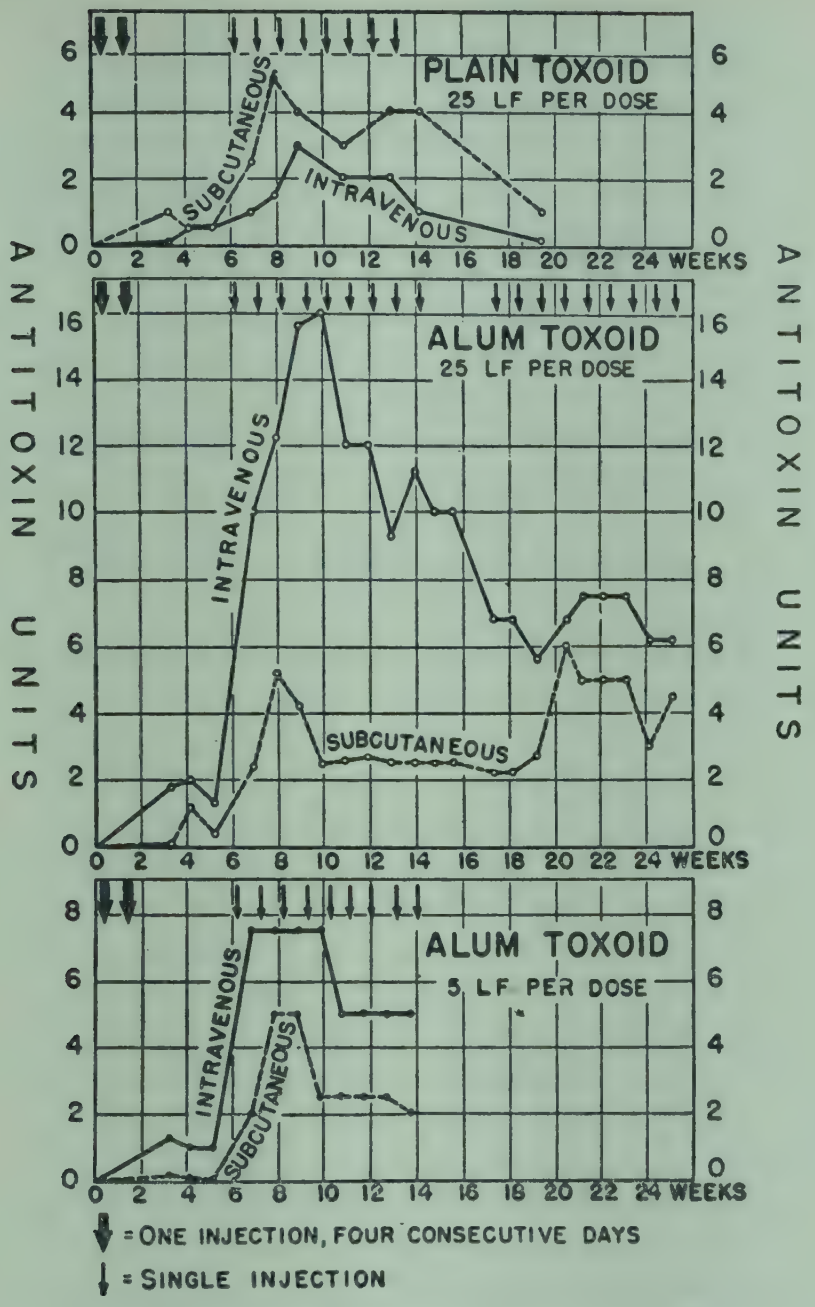

FIGURE 5. DIPHTHERIA ANTITOXIN FORMATION IN RABBITS
(From *J. Immunol.*, 1941, 40:437.)

antigen functions as a secondary stimulus. There is, however, no evidence for this assumption.

It is possible that a single injection of antigen, combined with an adsorbent, may act both as primary and secondary stimulus or in a way which is different from either. Antigens if combined with adsorbents such as alum injected subcutaneously produce higher and better sustained antibody response. Since at the site of the injection antigenic material is present for weeks, antigen is available for secondary stimulus. Holt (15) expressed the opinion that the single subcutaneous injection of fluid diphtheria toxoid induces a primary response and the single subcutaneous injection of alum precipitated toxoid serves both as primary and secondary stimuli. His opinion is based on the following. Primary antitoxin response to fluid toxoid has a latent period of two weeks, followed by a rise in titers for from about eleven to fourteen days and the maximum is sustained for twelve weeks. Alum toxoid, on the contrary, produces a peak in the curve at about the time when the animal is susceptible to secondary stimulation; removal of the depot four days after injection abolishes the peak. Whether the greater efficacy of alum toxoid can be explained solely by the assumption that it acts similarly to two widely spaced injections seems to be doubtful. Adsorption of an antigen on alum may have other effects. In this connection, it is of interest that in the rabbit fluid toxoid produces more antitoxin when given subcutaneously than when injected intravenously; alum toxoid, on the contrary, is more effective by the intravenous route (11). Landsteiner (16) believed that many factors may play a role in immunization with alum. They include slow adsorption, delayed removal of antigen, the particulate state, phagocytosis by antibody-producing cells and the stimulation of cell activity. Similarly in the adjuvant effect in immunization with a single injection of antigen in water-in-oil emulsion (paraffin) many factors may operate. Henle and Henle (17) called attention to the observation that a single injection of influenza vaccine in water-in-oil emulsion produces higher antibody titers than repeated injections of influenza vaccine in saline solution and that there is little antibody response to a second injection of influenza vaccine in salt solution. The question arises as to whether following a single injection of antigen in water-in-oil emulsion a new injection of antigen produces an additional immune response. When rabbits are given a single subcutaneous injection of formalin-killed bacilli in water-in-oil emulsion, a new subcutaneous

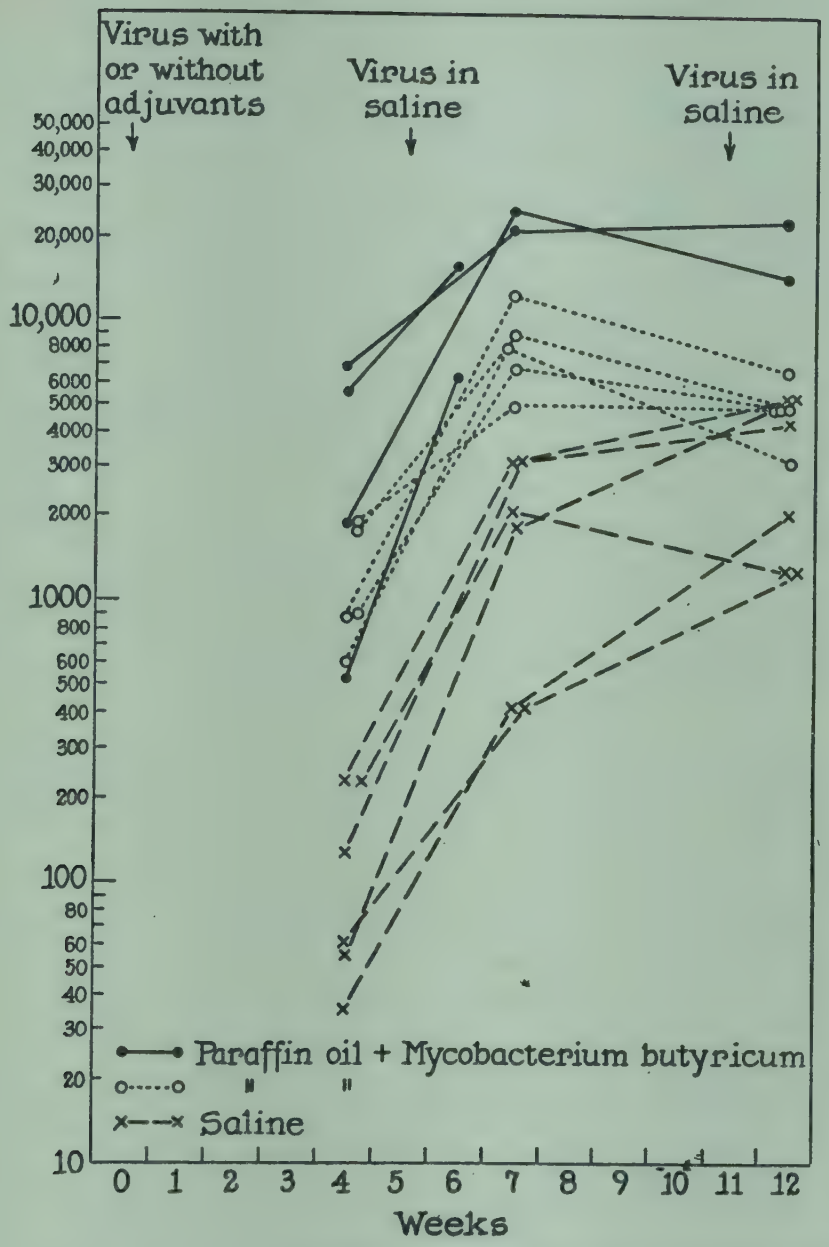

FIGURE 6. NEUTRALIZING ANTIBODY TITERS IN MONKEYS INJECTED WITH POLIOMYELITIS VIRUS WITH AND WITHOUT ADJUVANTS
(From *Proc. Soc. Exp. Biol. & Med.*, 1950, 74:536.)

injection of the antigen alone causes only a very slight response (18), but a second injection given intravenously produces a conspicuous rise in antibody titer (19). With other antigens, such as rabies virus or poliomyelitis virus, second injections bring about conspicuous secondary responses. Picrylated stromata of red blood cells combined with paraffin oil and killed mycobacteria injected intramuscularly produces skin sensitivity to picryl chloride; application of picryl chloride to the skin was found by Chase (20) to further intensify sensitivity. Since frequently repeated injections of antigen usually make animals almost refractory to a new administration of antigen, all these observations suggest that the effect of this adjuvant is not solely a subcutaneous depot effect with constant adsorption of antigen. The accumulation of mononuclear and epitheloid cells caused by this type of adjuvant may play a major part in its mode of action.

As it is not known how antibodies are formed, it is not surprising that the nature of the secondary response has not been elucidated. Recently three explanations have been offered. Holt (21) thinks the rise in antibody titers during the secondary response is due to a sudden release of stored antibodies. He bases his idea on the interpretation of the antitoxin curve during the secondary response of guinea pigs immunized with diphtheria toxoid. He believes that both the rise and fall in titer following the second injection are logarithmic. He argues that antibodies are released and not newly produced because in the primary response the ascending part of the curve is obtuse, not logarithmic. Holt thinks that the logarithmic character of the descending part of the curve of the secondary response is similar to the curve of antibody titers after passive immunization and this similarity is an additional argument for the idea that during the secondary response we are not dealing with new formations of antibodies. A comparison of the results with those published in the literature with reference to diphtheria antitoxin and other antibodies—see, for instance, Topley, Wilson and Miles (6) and Jensen (22)—shows that Holt's observations do not allow generalization.

Burnet and Fenner (23) offer another explanation for the secondary response. They emphasize the logarithmic phase of the rise in antibody titers during the secondary response in rabbits immunized with staphylococcus toxoid or bacteriophage. It is suggested that this phase is due to multiplication, at even rate, of an entity concerned with antibody formation. According to their hypothesis, the "function of the first antigenic

contact is to produce the adaptive modification (of enzyme-like units) while subsequent contacts stimulate their replication and eventual liberation of partial replicas into the bloodstream." This ingenious theory is supported by interesting analogies but not by direct evidence.

Haurowitz (24) suggests the following explanation: The secondary response is probably due to the combination of antigen molecules with intracellular antibody molecules in the antibody-producing cells. The penetration of antigen molecules into these cells has been proved by using isotopically labeled antigen molecules (Haurowitz and Crampton, *Science*, 1950, 111:300, and unpublished experiments). Most of the antigen molecules were found in the cytoplasmic granules of liver, spleen, and kidney cells. The intracellular combination of antigen and antibody molecules must cause localized precipitates and disruption of the cellular fine structure with subsequent release of antibody molecules from the damaged cells into the blood plasma, and increased compensatory protein production in the injured cells. Obviously, only soluble antigen molecules can penetrate rapidly into the antibody-producing cells. Particulate antigens will react slowly because their antigen molecules will be released slowly from the particles.

Non-specific secondary response.—Numerous reports in the literature describe the rise in antibody titer following non-specific stimuli. In the older German literature these were called the anamnestic reactions; some authors reserve this term for the specific secondary response.

Many of the observations were made so carefully and extensively that it is difficult to disregard them. There is no reason to doubt the observations of Weil and Felix (25) that during certain infectious diseases, such as typhus, lobar pneumonia and malaria, agglutinin titers against typhoid bacilli may rise in persons previously vaccinated with killed typhoid bacilli. Weil and Felix, during the First World War, tested the sera of a large number of persons ill with typhus for agglutinins against Proteus X_2 or X_{19} and typhoid bacilli. A part of the patients (soldiers) had been vaccinated against typhoid fever some time before admission. In about one half of the vaccinated patients agglutinins against typhoid bacilli were present when tested within the first six days of illness, and in one third of the patients these agglutinins appeared during the course of typhus. As a rule the titers rose conspicuously during the disease. About one fifth of the typhus patients who had not been vaccinated, but were from areas where typhoid fever was endemic, developed agglutinins

TABLE 1

AGGLUTININ TITERS AGAINST S. TYPHI IN TYPHUS PATIENTS PREVIOUSLY VACCINATED AGAINST TYPHOID FEVER

	DAYS OF TYPHUS			
	6	*10*	*12*	*15*
Titers against				
S. typhi	1000	1000	5000	1000
Proteus X_2	25	25	400	200

	DAYS OF TYPHUS			
	4	*9*	*21*	*38*
Titers against				
S. typhi	75	1000	1000	1000
Proteus X_{19}	2000	7500	2000	200

against typhoid bacilli. The Weil-Felix reaction was positive in all of these patients. Repeated cultures from blood, feces, and urine did not yield typhoid bacilli. It may be added that the sera of typhoid patients do not agglutinate Proteus X_2 or X_{19} strains and the sera of rabbits hyperimmunized with Proteus X_2 or X_{19} antigens do not clump typhoid bacilli.

The presence of typhoid agglutinins in the sera of persons previously vaccinated and the increase in titers was found not to be restricted to one disease, that is, typhus fever. They also occurred in tuberculosis, malaria, and lobar pneumonia, as mentioned above.

Similar observations were made by Conradi and Bieling (26) and others. Stimulated by these observations, Bieling (27) studied antibody formation in the rabbit after immunization with unrelated antigens. One typical experiment may be described briefly. Three rabbits received intravenous injections of killed Shigella dysenteriae. Two injections were given six days apart. Three weeks after the second injection, two rabbits were injected intravenously with killed Shigella paradysenteriae and the third rabbit with killed S. typhosa. Thereafter in all three animals there was a rise in the titers of agglutinins to the Shiga type of antigen (original injection). The rise was demonstrable within either one or two days after the heterologous stimulation. At this time it was slight, threefold or less. In all three rabbits there occurred a fall and a second rise in titer. The agglutinin formation toward the newly introduced antigen, either Shigella paradysenteriae or S. typhosa, appeared in five to seven

days. Bieling's experiments yielded fairly uniform results. He was not unmindful of the possibility of common antigenic components occurring in the strains used. Absorption experiments showed no common antigens between the two types of dysentery bacilli used in his experiments. There is no record in the literature showing that there are common antigens in dysentery and typhoid bacilli. The time aspect is most important

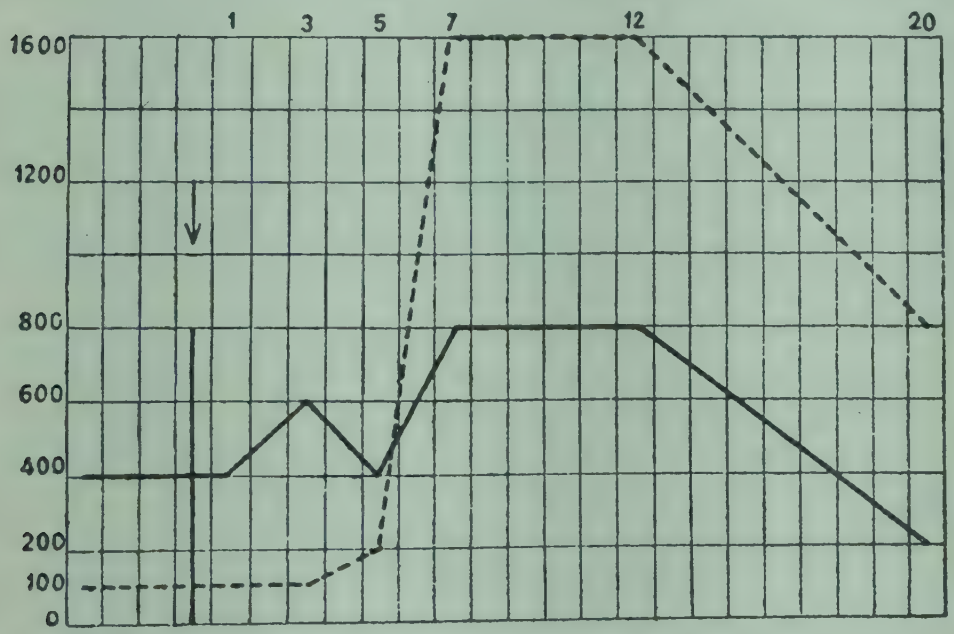

FIGURE 7. AGGLUTININ FORMATION AGAINST SHIGELLA DYSENTERIAE AND SHIGELLA PARADYSENTERIAE AFTER THE INJECTION OF THE LATTER IN A RABBIT PREVIOUSLY IMMUNIZED AGAINST THE FORMER
(From *Zeitschr. f. Immunitäts,* 1919, 28:246)
Ordinate: Continuous line, titer against Sh. dysenteriae; broken line, against Sh. paradysenteriae.
Abscissa: Time in days.
Arrow: Injection of Sh. paradysenteriae.

in Bieling's experiment. There is a rise in the primary antibody curve within one or two days and a fall and a rise again. This curve is so different from the specific secondary stimulus that it cannot be attributed to common antigens in the two species of bacteria used. The first part of the curve may suggest a release of antibodies.

Anamnestic reactions were described by Dreyer and Walker (28), who injected rabbits intraperitoneally first with killed *B. coli* and later with killed staphylococci. In this country, Mackenzie and Fruhbauer

(29) reported anamnestic reactions in rabbits immunized successively with four antigens, namely, horse serum, chicken serum, crystalline egg albumin, and S. typhosa.

There are reports (30) in the literature recording failures to produce non-specific anamnestic reactions. The positive and negative experiences are not entirely contradictory. As a rule, different workers used different antigens, time intervals, and methods of measuring antibody formation.

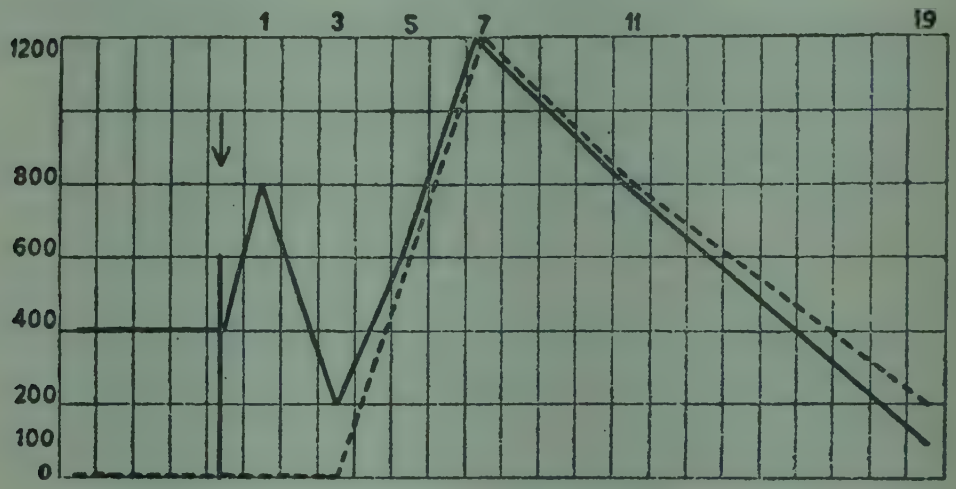

FIGURE 8. AGGLUTININ FORMATION AGAINST SHIGELLA DYSENTERIAE AND S. TYPHOSA AFTER THE INJECTION OF THE LATTER IN A RABBIT PREVIOUSLY IMMUNIZED AGAINST THE FORMER
(From *Zeitschr. f. Immunitäts,* 1919, 28:246)
Ordinate: Continuous line, titer against Sh. dysenteriae; broken line, against S. typhosa.
Abscissa: Time in days.
Arrow: Injection of S. typhosa.

A new type of anamnestic reaction was reported by White and Dougherty (31), who studied the effect of adrenotrophic hormone and adrenal cortical extracts on previously immunized animals. In one experiment rabbits were immunized with sheep erythrocytes and three months later, when circulating antibodies were no longer demonstrable, the rabbits were treated in the following way: Groups of rabbits were given a single injection of either adrenotrophic hormone or aqueous extract of adrenal cortex or cortical steroids in oil. According to White and Dougherty, antibodies appeared in the sera of animals within five hours following the injection of hormones; the titers rose to high levels

(ranging from 1:1000 to 1:4500 in individual rabbits) during the subsequent five hours and fell considerably during the following fifteen hours. The anamnestic response to the injection of sheep cells was hardly demonstrable 25 hours after injection. Following the injections of these hormones and concomitantly with the antibody response, the number of lymphocytes in the blood diminished considerably and there were striking changes in the lymphatic tissues: in lymph nodes, spleen, and thymus. The authors concluded that the hormones caused dissolution of lymphoid cells which released stored antibodies. Eisen, Meyer, Moore, Tarr, and Stoerk (32) excised the adrenals of rats and immunized them with killed pneumococci of Type I. Some of these rats received injections of adrenal-cortical hormones during the course of immunization. Antibody formation was measured by antibody N determination with reference to the pneumococcal specific carbohydrate. The administration of adrenal hormone did not influence the circulating antibody levels. On the other hand, an experiment with rabbits confirmed, in part, White and Dougherty's conclusions. Rabbits were immunized with pneumococci and later given a single injection of the hormone. There was a prompt rise of the antibody concentration of the sera in some, but not all, of the rabbits. The rise was about 30 percent expressed in antibody N and lasted for about twelve hours. It was concluded that cortical hormone does not influence antibody formation but causes the release of antibodies from the tissues into the blood. On the other hand, Fischel, LeMay, and Kabat (33) concluded that the cortical hormone does not affect the amount of circulating antibodies in immunized animals. They, like Eisen *et al.* (32), used antibody N determination, an accurate method but perhaps not sufficiently sensitive for this purpose.

Recently a group of French workers (34) confirmed, at least in part, White and Dougherty's report. They immunized groups of rats either with killed pneumococci or killed Salmonella enteriditis or sheep erythrocytes. One week after the last injection of antigen, half of the groups were given a single injection of aqueous extract of adrenal cortex. In one of the two groups of rats immunized with Salmonella enteriditis, the rise occurred in both the H and O agglutinins and began within four hours and lasted for one day. In a group of rats injected with sheep cells, the rise in titers was twofold and was noted eight hours after the injection of hormone. Electrophoresis and chemical analysis carried

out on pooled samples of sera showed no significant changes in the protein contents following the administration of cortical extract. The same authors in another paper (34) described their careful hematological and histological studies. In addition to the sharp decrease in the number of circulating lymphocytes, the most significant changes occurred in the spleen: (*a*) the perifollicular wreath of cells (Couronne perifoliculaire) diminished; (*b*) there was a proliferation of basophilic cells both in the follicles and in the red pulp; (*c*) in the centers of follicles large cells appeared with large foamy nuclei and with cytoplasm containing phagocytized material. Many of the proliferating cells could be classified as transitional and plasma cells in addition to the macrophages. These cells resembled those found by Fagraeus (35) two or three days after applying specific *secondary* antigenic stimulation. The authors felt that because the diminution of the number of lymphocytes in the peripheral blood and lymphatic organs occurred first and the proliferative changes later, the source of antibodies was the disintegrating mass of lymphocytes.

Perhaps the most striking aspect of the specific secondary response is the fact that it may occur a very long time after the introduction of the antigen and at the time when circulating antibodies are no longer demonstrable. Probably this is the reason for the French term "injection de rappel." The usual concept is that the second introduction of antigen induces antibody formation *de novo*. It seems more likely that the second injection of the antigen enhances the rate of antibody formation that is in progress. That antibody formation continues for a long time after certain antigenic stimulation is known to occur in yellow fever and some other diseases (human gamma globulin obtained from adults contains antibodies against the infectious agents of measles, mumps, and other diseases, as shown by Enders, 36). Under experimental conditions, Sugg, Richardson, and Neill (37) have shown that circulating diphtheria antitoxin can be found in guinea pigs for more than three years after antigenic stimulation; we (38) have observed that typhoid H agglutinins may be present in the sera of rabbits three years after injection of the antigen incorporated in paraffin oil. An illuminating observation was made by Doerr and Seidenberg (39) with reference to anaphylaxis. A female guinea pig was given a single injection of 0.002 ml. of horse serum. It is known that from 60 to 90 days after sensitization even a larger amount of serum of actively sensitized

TABLE 2

PERSISTENCE OF AGGLUTININ FORMATION FOLLOWING TWO SIMULTANEOUS INJECTIONS OF 0.25 ML. INTO THE SUBCUTANEOUS TISSUE OF RABBITS REPEATED AFTER SIX WEEKS

Group	*Killed Typhoid Bacilli*	*Salt Solution* (ml.)	*Aquaphor* (ml.)	*Paraffin Oil* (ml.)	*Killed Tubercle Bacilli* (mg.)
A	300,000,000	0.05	0.05	0.15	0.1
B	300,000,000	0.05	0.05	0.15	0
C	300,000,000	0.25	0	0	0

H AGGLUTININ TITERS THREE YEARS AFTER INJECTION

Group A	*Group B*	*Group C*
640	640	<10
	320	
	160	

animals does not transfer anaphylaxis passively. The guinea pig conceived in 230 days and gave birth to a litter 300 days after the injection of horse serum. The litter was found to be anaphylactic. It is most unlikely that this was the result of active sensitization of the fetuses, since immune response has not been shown to occur in embryonic life. The experiment seems to show that when circulating antibodies can no longer be demonstrated by the passive transfer of a few ml. of serum, the blood can passively sensitize fetuses by continued placental transmission of antibodies. Hence, circulating antibodies were present for more than 230 days after the injection of 0.002 ml. of horse serum. Since the half-life of passively transferred antibodies (precipitins against pneumococcal specific carbohydrate) is about two weeks (14), these considerations speak for a very long continued active formation of antibodies even when they are no longer demonstrable in the circulation.

The question arises as to whether a second injection provokes a secondary response if given very shortly after the first injection before antibody formation has occurred. Von Dungern (3) has shown a long time ago that a second injection of *maja* plasma given to rabbits two days after the first one has no effect on precipitin formation. All these observations lead to the view that the secondary response is very likely not causing antibody formation *de novo*, but enhancing specific antibody protein synthesis in progress.

It was shown by Opie (40) that when crystalline egg albumin is injected into the skin of a normal rabbit a large part of this antigen leaves the site of injection and appears in the blood stream; when a similar injection is made into the skin of a sensitized rabbit a large part of the antigen will remain in the skin. The fixation of antigen is specific and must be caused or mediated by the antibody. It seems probable that a combination of antigen and antibody will occur wherever they meet, in the bloodstream or in the tissues, and fixation may occur in many sites. It is possible that the antigenic effect of the combination of antigen and antibody globulin, made by the animal, is different from that of the antigen uncombined. It is very likely that fixation of the antigen to tissues is promoted. It has been known for a long time that foreign protein disappears faster from the blood in the immunized than in the non-immunized animal (von Dungern, 3). This does not imply that the foreign protein is eliminated faster in the immunized animal. On the contrary, it suggests that it is fixed more efficiently to the tissues of the immunized animal. The immune globulin originating in the host may give some protection to antigen against destruction or elimination. The combination of antigen with antibody may induce a more favorable cellular reaction or cellular activity than the antigen alone. It should not be overlooked, however, that adding antibody to antigen does not enhance antibody formation. The antibody may favor an advantageous, selective uptake of the antigen by functional cells.

It has been suggested that both the specific and the nonspecific secondary response is the result of a release of antibodies stored in certain cells, particularly lymphoid cells. White and Dougherty's report on the presence of antibodies in extracts of lymphoid cells several months after immunization lacks confirmation. While it has been shown by McMaster and Hudack (41) that lymph nodes draining the site of injection of antigen contain antibodies a few days after injection and before antibodies are detectable in the blood, later the antibody level is higher in the blood than in the lymph node. In a study (42) of the distribution of antibodies in the serum organs of rabbits either after active or passive immunization, the antibody titer of the serum was found to be higher than that of the organ extracts. It may be added that whole organs and not isolated cell types were extracted.

Chase (43) and others have shown that while the serum of an actively immunized (sensitized) animal fails to sensitize to tuberculin or simple

chemical compounds passively, cells from the spleen or lymph nodes may be effective. It has not been ascertained as yet whether the cellular transfer represents transfer of preformed, conventional antibodies or the antibodies are elaborated in the recipient animal by the cells received. Available evidence points to the latter alternative.

As long as there is no evidence for the storage of antibodies, it seems simpler to assume that in the specific secondary response we are dealing with enhanced antibody synthesis. In the non-specific secondary response the time aspect of the antibody response is suggestive of an enhanced release of antibodies. If it is difficult to prove that the secondary response is the result of a release of antibodies, it may be admitted that it is even more difficult to disprove it.

The assumption of a storage of specific gamma globulin is not in harmony with the present concept of the equilibrium between the proteins of the blood plasma and proteins of the tissues. Whipple and Madden (45) have shown the existence of a dynamic equilibrium between these proteins. On the one hand, after the removal of blood the concentration of blood protein is promptly restored and, on the other hand, a fasting dog can be kept in N equilibrium by the intravenous injection of dog plasma. Moreover, it was found (46) that by perfusion of the living immunized rabbit a larger quantity of antibodies can be removed from the immunized animal than presumably present in the blood before perfusion. Studies with isotopic amino acids have shown (47) that the proteins of tissues are undergoing a constant and rapid turnover.

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Chapter 4

FACTORS AFFECTING THE ANTIBODY RESPONSE

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Our knowledge of what we now refer to as antibodies undoubtedly began with the investigations of Héricourt and Richet, but it required the further studies of von Behring and Kitasato and of Ehrlich to place it on a sound and quantitative experimental basis. The findings of these workers gave support to the historical knowledge that recovery from many infectious diseases was followed by a marked resistance to reinfection, and to the specific demonstrations of the preceding twenty years that resistance could be induced by the inoculation of attenuated preparations of certain infectious agents. To be sure, there were times when the conventional concepts of antibody formation were challenged by such studies as the "Production of antitoxin by the passage of electricity through diphtheria cultures" (1). However, the last half century has, in general, seen a tremendous extension of our knowledge both of resistance and of antibody formation. We now can demonstrate the development of naturally or artificially acquired resistance to a great number of infectious diseases, in a very large proportion of which it has been shown that the resistance-conferring property or "antibody" is contained in the fluids which bathe the tissues of the resistant animal. Correspondingly, it has been demonstrated, in innumerable instances, that the introduction of any of a great variety of proteins, polysaccharides, and various other substances into the tissues of animals (including man) will likewise induce the production of antibodies in the serum and in the intercellular tissue fluids. Such sera will react specifically and more or less quantitatively with the inciting agent, and the antibody appears to have the properties of a specific serum protein. The quantitative determination of antibodies depends upon a variety of serological and immunological techniques, which have their limitations in precision and accuracy but without which our present knowledge would not exist. Thus, while recognizing the boundaries imposed upon our knowledge by these techniques, it is nevertheless appropriate to consider the avail-

able data regarding those factors which may affect the occurrence and the degree of the antibody response.

Since the days of Pasteur, many investigators have continued to approach the phenomenon of antibody formation indirectly through studies on resistance of the host to an infectious or toxic challenge. Such information, although not shedding direct light on antibody responses, is valuable, and will be referred to from time to time, especially in those instances in which studies of resistance are more pertinent and informative than studies of antibody levels. One need only consider, for example, the relative usefulness, in the study of experimental tuberculosis, of survival data as compared to titrations of any known antibody appearing in such infections.

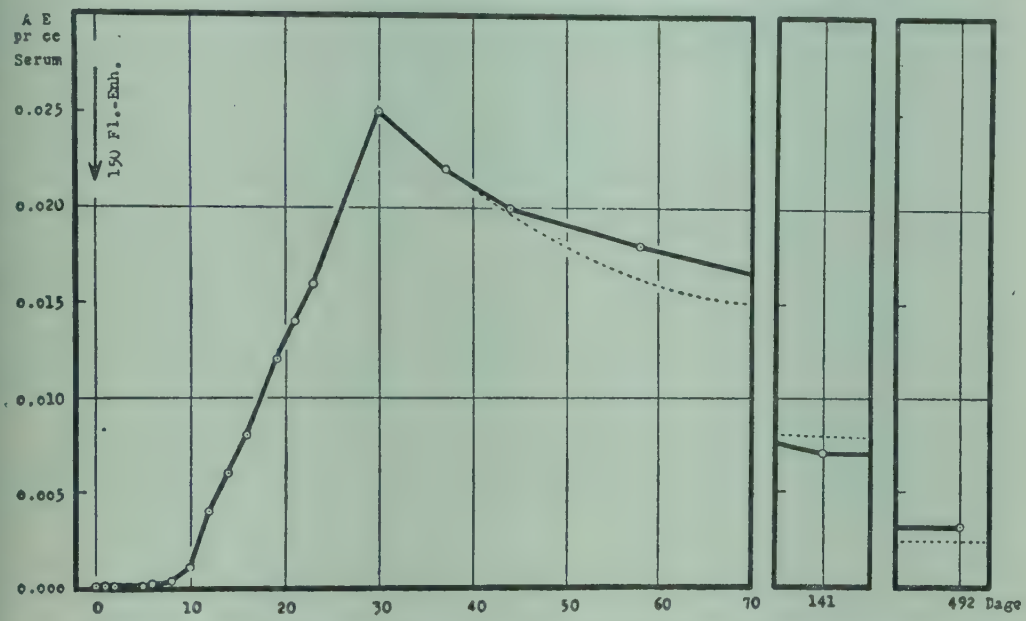
The general considerations, and much of the detailed factual knowledge regarding resistance, and antibody formation, have been extremely well set forth in the appropriate chapters of Zinsser, Enders, and Fothergill's classic monograph (2) and by Wilson and Miles (3). In the space here available, it will be possible only to touch on a few of the more important classical observations, and to bring in those more recent findings which seem most pertinent. The subject may be approached from two points of view: factors related to the stimulating antigen, and factors arising in the host. However, it is necessary, first, to establish certain generalizations regarding the antibody response.

PATTERN OF THE ANTIBODY RESPONSE

Those antigens which induce measurable antibody formation vary greatly in origin and in composition; they may consist of simple proteins, a variety of conjugated proteins, polysaccharides, or polysaccharide-protein complexes, and so on; examples are too well known and too numerous to cite here. Obviously, the response to all antigens will not be the same. With this reservation, however, the response to introduction of an antigen may be described in three phases: latent period, period of antibody increase, and period of antibody decrease. The length of the latent period, during which no detectable change occurs, appears to vary greatly with the antigen, the dose, the host animal, and other less well-defined factors. Although one may postulate that the variations observed are only a function of the variations in host, technique of immunization, technique of measuring antibody responses, and so forth

—in other words, that under uniform conditions the latent period for any effective antigen would be about the same—the available data do not permit a conclusion on this question. Hence the data are set forth below with a minimum of interpretation.

Burnet and his colleagues (4) have recorded the observed latent period in the following instances: In the rabbit, staphylococcus antitoxin appeared in about 8 to 13 days after a first injection of antigen; anti-



Effect of a Single Injection of 150 lf of Concentrated and Purified Diphtheria Toxoid in a 4-Year-Old Child without Antitoxin Prior to the Injection

(From C. Jensen *Acta path. et microbiol. Scandinavica,* 1933, 10:137)

rickettsial agglutinins in 2 to 5 days; herpes neutralizing antibodies in 7 days, and anti-phage antibodies in 3 to 4 days. Other investigators have observed the latent period in animals following inoculation of: *Salmonella schottmuelleri,* 6–8 days (5); horse serum, 10 days (6); and diphtheria toxoid, as little as 10 days if a very large dose is given (7). In the guinea pig, 100 Lf of fluid diphtheria toxoid may yield detectable antibodies in three weeks or less, whereas 1 Lf of precipitated toxoid has produced a detectable response in about two weeks (8). Diphtheria toxin-antitoxin mixture in the horse, like small doses of toxoid in the rabbit, may produce only a very slight primary response, or none at all (9). In man the same appears to hold true although a definite re-

sponse has been seen in as little as 10 days if a very large dose (for example 150 Lf units) is employed (10; see also accompanying figure).

The end of the latent period is marked by the appearance of detectable antibodies, which then rise more or less rapidly in titer toward a peak, or in some cases a plateau. The peak may be reached in from 2 to 8 or 10 weeks, depending on the animal, the agent employed, route of inoculation, and other factors. The rise appears, from the limited data, to be faster in the rabbit than in man or the guinea pig, and slowest in the horse; but as in observations on the latent period, studies using comparable doses per unit weight have not been found. Unlike the response to reinjection, for which Burnet (4) has described a logarithmic phase, the slope of the primary rise in antibody titer has not been shown to have any distinguishing mathematical characteristics.

The rate at which antibody levels decrease, after the peak has been reached, has been the subject of more detailed study, and it has many practical applications. A typical study is that reported by Fraser and Halpern (11) who observed the antitoxin titers in a group of children following a standard course of three injections of fluid toxoid.[1]

Expressed as percent of the titer at three months after the last injections, the subsequent levels were as follows:

Months	*Percent*
6	80
9	68
12	50
18	40
24	38
36	34

Such a prolonged maintenance of a significant antibody titer has been observed in many other studies, although some have reported more rapid declines. At all events, the falling phase of the curve is usually flatter than the rising phase, and it is generally assumed to be a composite of progressive antibody destruction plus continued antibody for-

[1] It may be objected that a series of three injections does not represent "primary" immunization. Technically this may be correct; however, in the absence of more precise data on true "one-dose" primary immunization, and also in view of the suggestive evidence (presented below) that the declining phase of a primary response may not differ fundamentally from that of a secondary response, it is felt that the data as given may be used as a basis for first approximations.

mation. The rate of antibody destruction may be at least approximated, from studies which have been carried out in man or experimental animals receiving transfusions of species-homologous antibodies, or in newborns acquiring maternal antibodies. Schoenheimer *et al.* (12), by feeding heavy nitrogen-containing amino acids to rabbits, gave evidence to suggest that the half-life of actively formed antibodies in these animals was about 14 days. Libby and Madison (13) have obtained a figure of six days for the disappearance of antibody to tobacco mosaic virus in actively immunized mice. Mason *et al.* (14) provided data which, if recalculated to allow for growth of the newborn animal,[2] indicated a half-life of about 28–30 days for neonatal, passively acquired antibody in lambs. Barr, Glenny, and Randall (15) and Wiener (16), working independently on different antibody systems, have obtained data indicating a half-life of about 30–31 days for maternally transmitted antibody in the newborn human. All observers seem to agree that the rate of loss of such antibody, passively acquired, proceeds as though it were a first-order process.

Certain of the data presented some years ago by Jensen (10) indicate that the rate of loss of actively induced antibody follows the equation for a second-order process:

$$kt = \frac{x}{a(a-x)}$$

where "x" is the amount of antibody lost, "a" is the amount of antibody at the start of the observation period, and "k" is a constant which is characteristic for, and usually different in, each individual. The experimental data offered by Jensen fit this formula remarkably closely (see Table 1). Furthermore, although most of his subjects—and particularly those with very high and easily measured antibody levels—undoubtedly manifested secondary or "booster" responses, it appears almost certain, from the nature of the data, that some of them were responding to diphtheria toxoid for the first time (see figure). The equation apparently fits both classes of subjects equally well. This correlation may well prove to be merely a coincidence; nevertheless, the finding is presented both for its theoretical interest and for its empirical value. Jensen employed it to predict the length of time during which a subject would remain immune to diphtheria. If the necessary titrations could be readily made, and the formula were to be found to have gen-

[2] I am indebted to Dr. A. S. Wiener (16) for this interpolation.

RATE OF DECREASE IN ANTIBODY LEVELS FOLLOWING ACTIVE IMMUNIZATION WITH DIPHTHERIA TOXOID

Comparison of Experimental Data with Predicted Levels According to the Formula:

$$kt = \frac{x}{a(a-x)}$$

	I.J., 8 years		J.N., 7 years	
t	a − x		a − x	
Days	*obs.*	*calc.*	*obs.*	*calc.*
0	20.0	20.0	9.0	9.0
37	9.6	9.7	1.4	1.2
73	6.3	6.5	0.55	0.58
185	2.9	3.1	0.22	0.24
342	1.9	1.9	0.13	0.13
380	1.7	1.7	0.11	0.11
731	0.98	0.97	0.05	0.06
	K = 0.0014		K = 0.023	

Source: C. Jensen, *Acta path. et microbiol. Scandinavica,* 1933, 10:137.

eral application, one can see the possibilities inherent in its more extensive use.

An unexpected consequence of Jensen's formula results from the fact that, in the second-order type reaction, the rate of destruction of antitoxin should be directly proportional to its concentration and to the constant "k." Thus, in the presence of very high concentrations of antitoxin—and particularly in individuals with a high "k" value—antitoxin may actually disappear at a rate faster than is seen following passive immunization. This surprising finding is borne out by Jensen's experimental data, one of his cases showing an antibody half-life, immediately following the peak level, which can be calculated as 4.3 days. This very high rate of antibody loss suggests that, in the presence of high antibody concentrations, there may be either (*a*) actual destruction of antibody as though it were a foreign body, (*b*) loss of activity due to an unusually unstable quality in such rapidly formed antibody, or (*c*) excretion of antibody through channels which are not ordinarily called into play. Obviously these findings require further study, and they, of course, also call for a careful reinvestigation of the data regarding the loss of passively acquired antibody, especially when very high initial levels are involved.

It has been well established, since the detailed studies reported by Glenny thirty years ago (9), that the antibody response in an animal already having had experience with an antigen is quite different from that seen on first contact with the same antigen. This second response, which follows a so-called "recall" or "booster" dose, is usually characterized by a much more rapid rise, a higher peak titer, and a more prolonged high level of antibodies. The characteristics of this secondary response will be taken up in detail elsewhere in this symposium (17) and it is sufficient to note here the inference, from Jensen's data, that the mathematical characteristics of the falling phase of the primary and secondary responses may be fundamentally similar, differing as a rule only in the quantitative levels concerned.

EFFECT OF DOSE OF ANTIGEN

In the majority of those infectious processes which confer immunity, there is abundant reason to believe that the efficacy of the immunizing process is related to the large amount of antigen which comes in contact with the antibody-forming tissues during the course of the infection.

It is when we are obliged to operate with inactivated organisms, or their inert chemical components, that we come imperatively up against the need—and likewise the opportunity—to consider the effect of dose of antigen on the immune response. Topley (5) has offered quantitative data to support the general finding of many investigators, that below a certain threshold dose of antigen, no *detectable* antibody response may appear. Such an observation must be interpreted in the light of the obvious limitations imposed on such studies by our methods of detecting antibodies. At any rate, it is well established, by various workers using experimental animals (5, 7, 10, 18, 19, 20), that as the dose of antigen is increased above a certain threshold, the measurable antibody response increases also. Numerous scattered observations have confirmed this for humans: for example, with diphtheria toxoid either as a primary or secondary stimulus (21–24); tetanus toxoid as a booster (25); or influenza virus presumably as a secondary stimulus (26, 27, 28). The last four observers cited, as well as McLean *et al.* working with swine (29), have reported data that are susceptible to quantitative analysis. In each case the increase in antibody response is very small in proportion to the increase in dosage of antigen, lying between the second and third root of

the increase in antigen employed, or even lower in one study (26). Edsall, Banton, and Wheeler (30) have recently carried out a similar study with diphtheria toxoid on about 150 medical students, decreasing the dose by fivefold increments, and have found approximately a second root relationship. Here again it is possible that there is no difference in principle between primary and secondary responses; as yet, however, the data are insufficient to rule this hypothesis in or out, particularly since true primary responses are relatively so feeble that they are difficult to study quantitatively.

It is obvious—again as noted by Topley (5)—that, beyond a certain point, increasing the dose of antigen will yield a proportionately diminishing return. In at least one instance, it is well known that an excess dose will actually inhibit the antibody response. This was observed more than twenty years ago by Schiemann and Casper (31), working with pneumococcus capsular polysaccharides, and it has been confirmed repeatedly since then. Felton (32) has shown that such large doses may prevent the animal from responding to subsequent doses of the same antigen for more than a year. He and others are agreed that this effect is related to long persistence of the antigen in the tissues of the host, as discussed elsewhere in this symposium.

CHEMICAL STATE OF THE ANTIGEN

Although it has been widely known for many years that many bacterial antigens were highly effective when prepared by certain methods, and were ineffective when prepared by other methods, it is not always possible to interpret such data with certainty. Obviously, a protein antigen which has been subject to prolonged high temperatures, or a polysaccharide which has been exposed to excessive acid or alkali concentrations, will generally be markedly or completely degraded. Subtler degradations have been observed, as illustrated by the removal of an acetyl group from the Pneumococcus Type I capsular polysaccharide (33, 34), in which the partially degraded antigen lost its efficacy for mice but not for men.

It is difficult to obtain concrete evidence on the quantitative changes in antigenicity resulting from slight alterations in the chemical state of the antigen; yet it is on such evidence that the evaluation of antigens and the definition of practical minimum standards for "good" antigens must

in large part depend. For at least one antigen—diphtheria toxoid—fairly concrete evidence is at hand regarding differences in the antigenic potency of preparations of equal "titer" as judged by flocculation. Numerous investigators have noted the occurrence of marked discrepancies between the immunizing and the flocculating potencies of certain batches of diphtheria toxoid, and several recent investigators (35–37) have stressed the correlation between flocculation *rate* and antigenic potency. The increase in antigenic efficiency per flocculating unit in purified as contrasted with crude toxoid which has been noted by various workers may, as has been suggested (37), be due to the removal of inhibiting substances or, as has also been suggested (38), to the removal of antigenically degraded but still actively flocculating toxoid molecules.

Reports on differences in antigenic efficiency, based on studies of preparations in clinical use, are plentiful, but difficult to interpret. It has clearly been shown, for example, that different batches of alum-precipitated diphtheria toxoid may induce "Schick conversion" in from as little as 20 percent to as high as 98–100 percent of children inoculated (39); but whether this variation was due to differences in antigenic quality, to differences in the technique of precipitating the antigen, or to other factors remains to be determined. There is thus much room for further studies on the properties of an antigen that determine its effectiveness in fulfilling the purpose for which it is used.

PHYSICAL STATE OF THE ANTIGEN: ADJUVANTS

It has long been known that particulate bacterial antigens are more effective, in horses or rabbits, when given intravenously rather than subcutaneously, and that the reverse is true for soluble antigens. This concept has been quantitatively confirmed by Freund (19) using fluid and alum-precipitated diphtheria toxoids in rabbits. The reasons for this may involve the nature and persistence of antigen depots, the rate at which antigen is absorbed, and various other factors, some of which are noted below.

The use of *adjuvants* probably antedates even the pioneer observations of Ramon, who had noted the enhancing effect of accidentally induced inflammation at the inoculation site, and who followed this up some years later (40) by incorporating tapioca into the antigen prepara-

tion. Subsequently Glenny and his colleagues (41) reported on the efficacy of alum precipitation of toxoids as an adjuvant procedure. This technique, or its modifications, using aluminum hydroxide (42) or phosphate (36) as adsorbing agents, has been almost universally adopted in the preparation of toxoids for human use, and has been applied to certain other agents such as pertussis vaccine. An even more potent technique, developed by Freund (43), is based upon the preparation of water-in-oil emulsions of the antigen, either with or without added killed tubercle bacilli. By this means, Freund has induced antibody levels which persisted, at reasonably high titers, for as long as 3½ years after two initial injections (44). However, the local reactions induced by antigens prepared in this fashion have so far precluded their routine use in man.

The mechanism by which such agents increase the antibody response has been disrupted. Glenny proposed that alum-precipitated antigens were retained, as depots, at the injection site, and he presented evidence contrasting the rate of loss of fluid vs. precipitated antigens (45). Holt (8) has shown that excision of the antigen depot within the first two weeks will lead to decreased formation of antitoxin, but later excision has little effect, presumably because encapsulation of the depot interferes with further antigen absorption. Freund and Lipton (46) on the other hand have shown that even very early excision of the depot does not abolish the effect of the antigen-adjuvant complex when a water-in-oil emulsion is employed, since there is prompt lymphatic absorption of the complex with probable secondary microscopic depots.

In this connection the effect on the antibody response of local reactions following inoculation has been remarked by many observers, with conflicting conclusions—mostly affirming, but some (47) denying such a relationship. The apparent conflict may perhaps hinge upon whether the reaction observed is essentially toxic or allergic in nature. Certainly there is evidence that local delayed allergic reactions around the inoculation site are associated with an adjuvant effect (48), and experimental evidence has been adduced to support the concept that inflammation (whether specific or non-specific) aids in retaining the antigen at the site of injection, and hence presumably exerts an adjuvant effect (49).

Combined antigens may be briefly considered at this point. There are many classical observations to indicate that two or more antigens administered simultaneously may mutually interfere with one another, and Björneboe (50) has furnished perhaps the best experimental evi-

dence for this. However, it is probable that most such findings were based on attempts to induce maximal antibody responses, such as in the production of potent antisera. It is understandable that in such instances the antibody-forming mechanism may well have been taxed beyond its capacity. By contrast, in recent years, numerous studies have shown that certain combinations of antigens—at least those in which one reagent is associated with local inflammation—may exert an adjuvant effect when employed in the doses customarily used for human immunization. Specific quantitative data to this effect have been adduced, in man and animals, using various antigens (51–54). On the other hand, there are observations which point the other way (55).

ROUTE OF ADMINISTRATION

Freund (19), as noted above, called attention to the influence of the intravenous vs. the subcutaneous routes on the efficacy of antigens in different states of solubility. Rowley (20) has shown that the difference in response observed with these routes may be a reflection of the role of the spleen in formation of antibodies, since removal of the spleen largely abolishes the difference in response ascribed to these two routes.

The *intradermal* route has received extensive but not always strictly quantitative study. Wishart (56) has critically evaluated this route for diphtheria immunization and found it inferior to the subcutaneous route. However, in this case, as in most others, it is difficult to make comparisons, since the dose given intracutaneously is almost invariably less than that given subcutaneously.

For typhoid vaccine, conflicting results are at hand, as noted in a previous review (57), and for many other agents the extensive data available are generally inconclusive (57, 58). Nevertheless, this route offers certain potential advantages. As several investigators have noted (58), for such agents as influenza vaccine it may provide an adequate antigenic stimulus with a lower incidence of reactions than that observed after subcutaneous injection of standard doses. A modification of the intracutaneous approach—the "jet" technique reported a few years ago (59)—will be of interest to watch for reports on its comparative efficacy; recent studies on its use (60) suggest that it provides the basis for an effective immunological stimulus.

Much has been attempted with the *intranasal* route, but little has been

accomplished by way of primary immunization, although it appears highly efficient in providing a *secondary* stimulus to antitoxic antibodies (57). Much the same observations can be made regarding the *oral* route, which has been comprehensively reviewed recently by Dolman (61). Various other routes might be considered here—percutaneous, inhalatory, or even peranal—but few have been sufficiently explored to justify generalization.

INTERVAL BETWEEN DOSES

Related to the prevailing concepts of the mechanism of action of adjuvants is the influence of spacing of doses on the antibody response. Carlinfanti (62) has recently shown that a given quantity of antigen is more effective if given in divided daily doses over a period of several days, than if given in one inoculation. However, when the interval is lengthened to about two weeks (in man; the threshold interval is probably shorter in smaller animals), the characteristic secondary response begins to appear with the administration of the second and subsequent doses; and, in general, longer intervals up to perhaps several months (again, in man) increase the magnitude of this response, provided that adequate primary immunization has been established. From a practical point of view in human immunization the gain in response which results from employing intervals longer than one week has been recognized since Ramon called attention to it in 1927 (63), and it has been confirmed by others (64) with diphtheria toxoid in man and with bacterial vaccines in animals (65) or in man (47).

HOST FACTORS

Many of the host factors related to immunity have been studied largely in relation to resistance, rather than in terms of antibody formation. Thus, there is a large body of information relating to species resistance (or susceptibility) to a great variety of infections (2, 3). Strain differences in resistance have been subjected to extensive and thorough study both in mice (66) and in rabbits (67). Some observations indicate differences in capacity to form antibodies, correlated with family strains, in guinea pigs (68) or rabbits (69), and it is possible that the strain difference in susceptibility to reversed passive anaphylaxis in guinea pigs,

reported by Zinsser and Enders (70), was a related phenomenon. But the factual data are few and far between. Furthermore, any such data would have to be reexamined from a statistical standpoint, since individual animals differ widely, not only in their resistance to infection (71) but in their antibody response to a given antigen stimulus. Carlinfanti and Cavalli (72) have shown that the distribution of such responses, in a randomly selected population, follows a normal, Gaussian distribution curve, and the data of Stewart and Jones are not greatly dissimilar (73). This at least provides a basis for evaluating any findings obtained under more varied conditions.

The *age of the host* is known to play a role in the antibody response. Moll (74) foreshadowed this long ago. Since then Freund (75) and various others have shown that young mammals are poor antibody producers, and Wolfe and Dilks have made similar observations in chickens (76). However, such findings can no longer be regarded as proof that immunization in the neonatal period is ineffective or impractical, as has been commonly believed. Numerous studies within the last few years have shown that effective immunization against pertussis and tetanus in infants three months old or younger can readily be achieved, and that the same appears to be true of diphtheria in those infants who have not passively acquired maternal immunity. Many of these studies have been summarized in a recent review (58); others, pointing to the same affirmative conclusion, continue to appear (77–79).

Related to these considerations on neonatal anti-diphtheria immunization, there continues to be some interest in the problem of so-called *combined active-passive immunization*—the question again hinging on the influence of passive antibody in retarding the action of a corresponding actively introduced antigen. The practical problem usually arises when an individual, not immune to tetanus (or diphtheria), must receive an emergency prophylactic dose of antitoxin but should also be started, as soon as possible, on active immunization. The prevailing evidence, indicating how this can successfully be accomplished, has been summarized in a recent editorial (80). It is interesting to note that the action of antigen is not inhibited by passively acquired antibody only; recent observations (30) suggest that high levels of *actively* acquired antibody may have the same effect.

INJURY TO THE HOST

Space does not permit more than passing consideration of this extremely broad topic. Pages have been written on the effects of various dietary deficiencies on resistance and immune responses (3). A recent and exhaustive review deals with the effect of X-radiation on immunity (81). Cannon (82), along with numerous others, has amassed an impressive body of evidence pointing to a close correlation between protein deficiency and impairment of the antibody-forming function; and Balch (83), on the other hand, has reported extraordinarily high antibody responses in extremely cachectic individuals. The effects of cold, heat, and other changes in physical environment have intrigued investigators ever since Pasteur and his colleagues first gave experimental evidence of the effect of chilling on susceptibility to infection.

Hormonal Influences. The effect of hormonal activity on antibody levels has attracted unusual interest in recent years, particular attention being given to the reported ability of adrenal cortical hormone to elevate circulating antibody levels. The earlier observations of Fox and Whitehead (84) to this effect were confirmed and extended by the studies of Dougherty, Chase, and White (85, 86), but have been questioned by several subsequent investigators, who have found either no change in antibody level, or a fall in titer, following adrenal cortical stimulation (87–89). These conflicting studies have recently been reviewed (90, 91); since these reviews appeared, further studies have added to the negative evidence (92–94), and one investigation, involving administration of cortisone to experimental animals from the onset of immunization, has shown complete suppression of antibody formation thereby (95). Thus there are many ramifications to the problem, and the overall question is still *sub judice.*

CONCLUSION

Evidently the antibody response is influenced by a great variety of unrelated factors, and much remains to be learned about most of them. It is also apparent that continued study has, in several instances at least, reversed the established opinion on points which were previously regarded as thoroughly settled. From a practical point of view, it would be pleasant to be able to conclude with a definition of what constitutes a

"good" antigen—that is, what constitutes "antigenic potency"; for this is what is sought not only in most experimental studies but also in the practical application of the principles of immunity to the welfare of animals and man. Obviously, however, such a definition is still elusive. From the data presented above, it is apparent that so many variables enter into any study of antigenicity that comparable observations, with all variables controlled but one, are extremely difficult to achieve even in experimental animals, to say nothing of the difficulties they present in man. It should also be stressed at this point that the correlation between such laboratory findings and their practical applications is frequently imperfect. One may cite, for example, the employment of agglutination or mouse protection titers in evaluating the "potency" of typhoid and pertussis vaccines in man, as instances of laboratory tests which in themselves can be performed with considerable accuracy, yet which give information that has not to date been adequately shown to correlate with antigenicity as measured by protection of humans against clinical infection.

It is, of course, to be expected that many of the existing problems with regard to antibody formation will be resolved in the not too distant future, although further questions will, with equal certainty, arise along lines which we cannot at present foresee. Our reactions to the available data will alter as further evidence accumulates, and thus we will become allergic to these problems, in the sense that von Pirquet used "allergy" to mean "changed or altered reactivity." If the above discussion has in this respect functioned to some extent as an adjuvant, it will have served its major purpose.

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Chapter 5

PERSISTENCE OF ANTIBODIES IN MAN AFTER IMMUNIZATION

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Active immunization of human beings has been practiced for two centuries, but it is only in the last few years that we have learned anything of the actual quantities of antibodies formed as a result of the injection into man of even a limited number of bacterial antigens. This lag has been due to the far smaller quantities of antibodies ordinarily produced by vaccination as compared with the amounts engendered in the hyperimmunization of animals. Microanalytical methods which had given exact knowledge of the quantities of antibody, in weight units, in the sera of hyperimmunized animals (1) proved too insensitive for direct application to the sera of immunized human beings. Quantitative methods were, however, eventually adapted to the study of human sera (2) and a considerable body of data is now available (3–6). It is the purpose of the present paper to discuss the antibody levels disclosed by some of these data, as well as the persistence of the antibodies formed, the effects of booster doses, and the bearing of the findings on current theories of antibody formation.

DIPHTHERIA ANTITOXIN

Accurate measurement, in relative terms, of diphtheria antitoxin formation in man has long been possible by titration in the rabbit skin (7) or by the Ramon flocculation test. After the isolation of highly purified diphtheria toxin and toxoid (8) it was feasible to apply the quantitative analytical chemical methods for the estimation of antibodies to the determination of diphtheria antitoxin in weight units, first in horse sera (9) and then in the sera of man and other animals (5). By use of the value 1 antitoxic unit = 2.6 μg N (5) it has been possible to recalculate early data (7) on the antitoxin content of the sera of children who had received a single injection of 150 Lf (about 0.7 mg.) of diphtheria toxoid.

TABLE 1

MICROGRAMS ANTITOXIN N PER 4 ML. SERUM AFTER 150 LF TD

Initials of Child	*Maximum ca. 2 wks.*	*1 mo.*	*2½ mos.*	*4½ to 6 mos.*	*17 mos.*	*2 yrs.*
T.M.	1870	...	...	...	...	...
E.N.[a]	1040	260	125	42	21	...
I.J.	208	104	66	31	...	10
J.N.	94	16	7	2	...	0.5
L.O.	10	3	2	1		
K.F.	3	...	2	...		

					AFTER REINJECTION		
					1 wk.	*1 mo.*	*3 mos.*
G.K.	0.6	...	...	...[b]	33	11	5

0.05 AU/ml., or 0.2 AU/4 ml. = 0.5 μg AN

[a] Rec'd 250 Lf Td, 0.7 mg. Td. [b] Reinjected with 0.5 Lf Td, 1.2 μg Td.

In Table 1 and Figure 1 these values are expressed as μg of antibody nitrogen per 4 ml. of serum, the basis of comparison used for the sera of individuals injected with pneumococcus polysaccharides. One notes the rapid increase (dotted lines, Fig. 1) to a maximum in about two weeks and the rapid decline during the next two weeks to about one half or less of the maximum value, followed by a slower decrease during the next months and years. Jensen, whose skin-test values are here translated into weight units, calculated the decrease on the basis of a bimolecular reaction, and found that attainment of the minimum protective value of 0.05 antitoxic unit might often take 10 to 60 years in spite of the rapid initial rate of decrease. The curves shown in Figure 1 are those of three of the four best antibody formers in the series, with 1040, 208, and 94 μg of antitoxin nitrogen per 4 ml. at the peak; the fourth, with 1870 μg N, is omitted because no subsequent values were given. In Cohn and Pappenheimer's series (5) the antitoxin content of the sera of adult subjects ranged from 52 to 3750 μg N per 4 ml.

ANTIBODIES TO THE TYPE SPECIFIC POLYSACCHARIDES OF PNEUMOCOCCUS

It has been shown by numerous examples that subcutaneous injection of about 50 μg each of two to six type-specific polysaccharides of pneumococcus into man is usually followed by the formation of type-specific

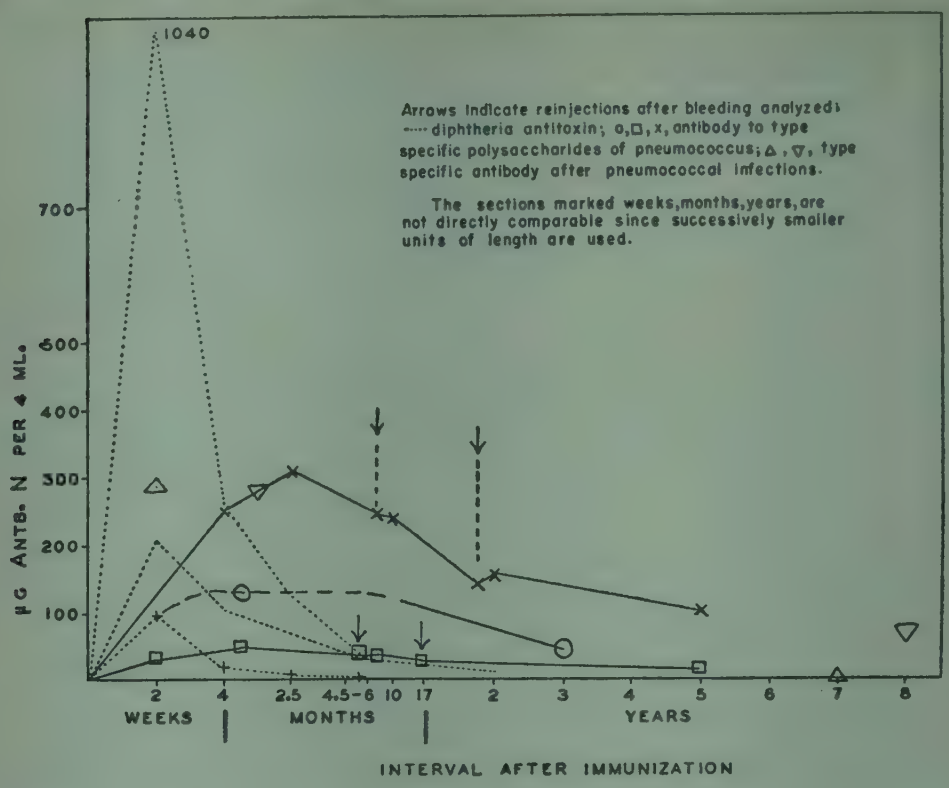
1040
700
600
400
300
200
100
μG ANTB. N PER 4 ML.
Arrows indicate reinjections after bleeding analyzed; ----- diphtheria antitoxin; o,□,x, antibody to type specific polysaccharides of pneumococcus; △,▽, type specific antibody after pneumococcal infections.
The sections marked weeks, months, years, are not directly comparable since successively smaller units of length are used.
2 4 2.5 4.5-6 10 17 2 3 4 5 6 7 8
WEEKS
MONTHS
YEARS
INTERVAL AFTER IMMUNIZATION

FIGURE 1. MICROGRAMS OF ANTIBODY NITROGEN PER 4 ML. SERUM OF HUMAN SUBJECTS AT INDICATED INTERVALS AFTER IMMUNIZATION

antibodies which rise to peak levels in two to six weeks (3b). Contrary to the behavior of antitoxin in the human subject, which drops off to one half or less during the second two-week period and continues to decrease at a slower rate, the type-specific anticarbohydrate in human sera remains at or near the top level for 5 to 8 months (3b; see also Table 2) and then slowly decreases. In two groups of volunteers whose sera were analyzed 3 years (Table 3) and 5 to 6 years (Table 4) after the original injection, antibody values ranging from one fifth to one half or more of the maximum persisted in spite of the long interval (3f; see also Fig. 1, solid and dashed lines). A few subjects were found who had received Types I, II, and III pneumococcal polysaccharides 8 years previously, and these also showed abundant residual antibody (Table 5) in proportion to the mouse protection values originally noted (3f).

Table 2

Antibody Levels in Human Beings after Subcutaneous Injections of Specific Polysaccharides of Types I, II, and V Pneumococcus

Subject No.	Antibodies to	micrograms antibody N per 4 ml. serum						
		Bleeding No.						
		0	1	2	3	4	5	6
		Before	*2 wks.*	*6 wks.*	*5½ mos.*[a]	*8 mos.*	*15½ mos.*[a]	*4–5 mos. later*
79	C				43	75	69	73
	I	0	7	7	14	9	2	4
	II	0	14	28	29	26	19	⩾19
	V	1	28	36	35	30	19	
80	C				12	25	32	34
	I	6	72	63	73	54	45	39
	II	1	37[b]		18	12	11	10
	V	1	129	136	166	143[c]	97	<113
81	C				17	15	26	24
	I	1	15	21	17	14	10	10
	II	1	23	29	12	12	10	
	V	0	1	2	5	1		
82	C				74	41	63	68
	I	3	14	16	7	10	4	3
	II	1	67	78	50	44	24	24
	V	1	74	79	57	51	24	

Source: M. Heidelberger *et al., J. Exp. Med.*, 1946, 83:303.

[a] Followed by reinjection of 50 μg each of S I and S II, except that after bleeding 5 No. 80 received 50 μg S V in addition.

[b] In order II, V, I. Order V, II gave anti-V, 139; anti-II, 31.

[c] By micro-Kjeldahl:146.

Table 3

Persistence of Antibodies in Human Sera after Injection of Type Specific Polysaccharides of Pneumococcus

four typical cases in a series of eleven

Antibody to:	C	I	II	III	V	VII	VIII
Subject							
83_0	16	0	0		0	3	
1	14	21	128		14	130	
2	17	9	44	0	3	86	1
3	15	6	37	12	1	78	27
86_0	25	0	0		0	7	
1	25	6	29		0	61	
2	39	3	6	1	0	29	0
3	35	5	8	15	0	35	2
91_0	37	0	0		1	14	
1	42	55	25		7	151	
2	45	19	15	3	0	73	1
3	71	25	22	59	0	73	49
94_0	77	1	0		0	10	
1	89	70	36		0	18	
2	21	14	9	0	0	2	1
3	46	20	13	16	1	0	44

Source: M. Heidelberger *et al., J. Immunol.*, 1950, 65:535.

0 bleeding taken before injection of specific polysaccharides of Types I, II, V, and VII pneumococcus.

Bleeding 1, two months later.

Bleeding 2 taken 3 years later and followed by injection of specific polysaccharides of Types I, II, III, V, VII, and VIII pneumococcus.

Bleeding 3, two months later.

TABLE 4

FORMATION AND PERSISTENCE OF ANTIBODIES IN HUMAN SUBJECTS INJECTED WITH PNEUMOCOCCAL POLYSACCHARIDES

Reinjection after 5 to 6 years; data on earlier bleedings in *J. Exp. Med.*, 1946, 83:303

ANTIBODY TO:	C	I	II	III	V	VII	VIII
Subject and Bleeding No.	*Micrograms Antibody Nitrogen per 4 ml Serum*						
107	10	3	17				
8	18	1	16	0	0	1	2
9	13	5	15	4	0	32[3]	19
524	7	4	48				
5	23	0	22	1	0	0	3
6	24	13	14	99	0	36[8]	10
616	77	2	0		2[a]		
7	77	3	0	0	0	0	0
8	83[4]	0	0	3	2	0	4
655	20	9	27		4[a]		
6	30	4	15	0	0	0	1
7	31	8	19	8	6	0	7
706	67	14	28		3[a]		
7	48	7	19	2	0	1	0
8	44	9	17	3	7	28[5]	17
765	109	76	29		9[a]		
6	96	67	14	2	1	14	0
7	133	68	21	77	2	38	97
826	68	3	24		51[a]		
7	70	1	16	2	0	0	0
8	66	2	15	0	20	36[8]	13

[a] About two years earlier than the other values recorded.

Source: *J. Immunol.*, 1950, 65:535.

NOTE: Values on the first line of each set are from the last bleeding 5 or 6 years previously. The second line gives the analytical data before the injection of S I, II, III, V, VII, and VIII; the last line, values 2 months later.

TABLE 5

FORMATION AND PERSISTENCE OF ANTIBODIES IN HUMAN SUBJECTS INJECTED WITH PNEUMOCOCCAL POLYSACCHARIDES, TYPES I, II, AND III

Reinjection[a] after eight years

ANTIBODY TO:	C	I	II	III	V	VII	VIII	I MOUSE PROTECTION 1941
Subject and Bleeding No.[b]	*Micrograms of antibody nitrogen per 4 ml. serum*							
403_{n+0}	50	0	6	0	0	0	0	0
n + 1	50	71	7	2	13	1	28	
n + 2	36	49	11	1	10	0	23	
404_{n+0}	67	72	51	0	0	4	5	++
n + 1	65	71	53	6	5	11	6	
n + 2	61	68	57	7	6	10	8	
405_{n+0}	14	8	12	17	0	0	1	+
n + 1	16	14	23	22	6	11	8	
n + 2	17	13	25	23	8	10	9	
406_{n+0}	13	16	2	5	0	0	0	+
n + 1	13	20	13	9	23	36	21	
n + 2	11	19	11	6	13	30	14	
407_{n+0}	33	59	27	0	0	21	4	
n + 1	37	58	27	9	3	23	22	
n + 2	40	63	29	9	4	24	17	

Source: *J. Immunol.*, 1950, 65:535.

[a] Subcutaneously with 1 ml. Squibb's solution of 50–70 μg each of the specific polysaccharides of Types I, II, III, V, VII, and VIII pneumococcus.

[b] Bleedings n + 0, n + 1, n + 2 before, 2 weeks after, 6 weeks after reinjection, respectively.

COMMENT

Not only does the type-specific pneumococcal anticarbohydrate persist in man at relatively higher levels over a longer period of years than does diphtheria antitoxin, but there is another fundamental difference between these antibodies. It is well known that when diphtheria antitoxin has sunk to a low value, a small "recall" or "booster" dose may cause the titer of antitoxin to rise rapidly to many times its original maxi-

mal value. This is well illustrated by the last case in Table 1, in which the serum attained a value of only 0.6 μg of antitoxin N per 4 ml. in two weeks. Two and a half months later, however, following a very small reinjection of toxoid, the quantity of circulating antitoxin rose within one week to 33 μg N per 4 ml. and then decreased at about the rate expected after a primary injection. In the case of pneumococcal anticarbohydrate, booster effects of comparable magnitude were not noted. In general there was little change following reinjection of polysaccharide, especially when an appreciable level of circulating antibody remained. Often when the antibody to a polysaccharide had almost disappeared a measurable level could be restored by the reinjection, but the height attained was rarely that of the original maximum. Numerous instances will be found in the tables.

We have, then, in a single animal, man, two kinds of accurately measurable antibodies which show divergent behavior. On the one hand is the antitoxic type which rises rapidly to a maximum, recedes within a few weeks to much lower levels, and can be recalled, often to far higher values, by further injections. The other variety is exemplified by the antibodies to the type-specific polysaccharides of pneumococcus, and is characterized by a possibly slightly slower rise, a long plateau at or near the maximum lasting 5 to 8 months, and a slow decline over a period of years; in this period, reinjections of polysaccharide have little effect on existing antibody levels. How can these radical differences be accounted for, and how adequately do current theories of antibody formation explain these well-documented and experimentally firmly established divergences of antibody behavior? A tentative explanation can be based, it is believed, upon the chemical properties of the antigens under consideration and upon the current theories of antibody formation.

Toxins and toxoids are typical proteins: so much so, in fact, that nothing has yet been found in their make-up of amino acids to set them apart from other proteins and account for their highly dangerous properties. Like other proteins, diphtheria toxin, for example, is destroyed by proteolytic enzymes (8).

Insofar as the pneumococcal polysaccharides have been studied, these substances appear to be extremely resistant to attack by the ordinary carbohydrate-splitting enzymes. Saliva, for example, is a valuable aid in removing starch or glycogen from crude polysaccharides during their isolation from culture fluids (10). The writer will never forget his aston-

ishment at an early demonstration of this indestructibility. He and Dr. O. T. Avery made up a slurry of filter paper pulp in a solution containing about 1 part in 20,000 of the specific polysaccharide of Type II pneumococcus and seeded it with thermophilic cellulose-destroying bacteria obtained from Drs. Peterson and Fred. After a night at 55° C. the filter paper had disappeared, but serological tests showed that the mixture still contained about 1:20,000 Type II polysaccharide! These substances, then, are not as easily disposable as are the toxins. Let us see how the theories of antibody formation may be adjusted to the limitations imposed by these properties.

The two theories of antibody formation most widely supported at the present time are those due to Breinl-Haurowitz-Mudd-Alexander-Pauling and to Burnet. In the former theory, antigen or an immunologically active fragment penetrates to a site of globulin formation and there by its presence distorts the synthesis in such fashion that the new globulin molecules emerge so altered as to be able to react with the antigen when it is again encountered. In the Burnet theory the presence of the antigen is required only long enough to change in some characteristic way the enzymes or framework upon which the globulin is built. After that the antigen may disappear, but the altered globulin, or antibody, continues to be synthesized. This theory has the advantage of accounting for the rapid secondary outpouring of antibody after the enzymes have been "trained" by the initial injection.

Let us assume that these theories are not mutually exclusive and that both methods of antibody formation might operate simultaneously or that the Breinl-Haurowitz theory might be the initial stage of antibody production even under the Burnet hypothesis. At the start the presence of the antigen or its immunologically reactive fragment will be the dominating factor while the enzymes are being "trained." If the antigen is a toxoid or protein one would expect it to disappear rather rapidly by proteolysis so that one might observe an initial rapid rise during the first two-week period, followed by a rapid fall as the direct stimulus to antibody production is digested away and the indirect mechanism takes over. When and if all of the antigen has disappeared by proteolysis this mechanism would account for the continuing formation of antitoxin at gradually lower and lower levels as the enzyme or framework molecules, which themselves have only a limited life, slowly diminish in number. A reinjection during this period would rapidly renew the cycle.

The events following injection of an indestructible antigenic polysaccharide would be somewhat different, for the first stage could and perhaps does go on almost indefinitely. Antibody production, stimulated by the direct presence of the antigen, rises almost as rapidly as in the case of toxoid, to approximately similar levels. Since there is no destruction of antigen, production of antibody continues for months at the maximum. If a cell dies, polysaccharide is released and attaches itself to a new globulin-synthesizing cell. There is, perhaps, a limited degree of "training" of globulin-synthesizing enzymes, but this effect is not needed if most of the antigen remains intact to stimulate the organism to its maximum antibody-synthesizing effort. In this event a booster dose would have little effect, as is actually observed (Table 2; Fig. 1, arrows).

The foregoing suggestions, while admittedly rather speculative, at least have the virtue of resting upon a large body of accurate analytical data, upon the known biochemical properties of the substances involved, and upon the currently accepted theories of antibody formation. Their main weakness lies not in the solid experimental data upon which the suggestions are based, but rather in the theories of antibody formation. Although these have their elements of plausibility and account at least partially for many observed phenomena, neither is wholly satisfactory nor rests upon any direct experimental foundation. Possibly the new suggestions and data may help to reconcile the two theories and to point the way to badly needed experimental tests of their validity.

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DISCUSSION

DR. PAPPENHEIMER: We have heard a good deal about the secondary response this evening. In their recent book, Burnet and Fenner have criticized prevailing theories of antibody formation on the grounds that they all fail to explain the great difference in antibody production following secondary as compared with primary stimuli. I must say that Burnet and Fenner's hypothesis that antibody formation can take place in the absence of antigen does not make these phenomena any clearer to me.

DR. HAUROWITZ: Our investigations on the intracellular distribution of injected antigens have shown that the large antigen molecules penetrate easily the cellular membranes of liver cells and pass into the cytoplasm and the nuclei of these cells. If this happens in a sensitized animal, the injected antigen will react with intracellular antibody molecules. This intracellular antigen-antibody reaction may produce damage and disruption of the intracellular structures, and may thus cause release of antibody from the cells.

DR. PAPPENHEIMER: I wonder whether the presence of small amounts of antibody in the cell might not enable it to capture antigen more easily?

DR. W. E. EHRICH: I think we must distinguish between reticulo-endothelial cells and reticulum cells as regards antibody formation. There is no evidence that reticulo-endothelial cells are concerned with antibody production. However, reticulum cells can differentiate to lymphoid cells, the only cells from which antibodies have actually been extracted. Attempts to extract antibodies from thymus lymphocytes have failed. Aggregations of plasma cells produced by hyperimmunization, on the other hand, yielded high antibody concentrations. The latter observation is in agreement with the clinical experience that hyperglobulinemia is associated with a rise in plasma cells, but there is no hyperglobulinemia in lymphocytosis or lymphatic leukemia.

Dr. McMaster: I feel that it is most important to have better definitive descriptions of cell types, as has just been pointed out by Dr. Ehrich. It will be necessary to have cytologists agree upon just which cells are to be regarded as plasma cells and which are not, which are lymphoid but not lymphocytes, and what is the relationship of these cells to one another and to reticulo-endothelial cells. Until then confusion will prevail.

Dr. William Boyd: There is one interesting point which has been observed by a great many workers and which is worth emphasizing. If one starts immunizing rabbits with a single antigen and then continues to add a new antigen each week, one finds a good response to the antigens used at first but eventually the response becomes less. One wonders if the antibody-forming mechanism can become fatigued?

Dr. Pappenheimer: When many antigens are used in this way does one find an increased number of cross reactions? In other words is there any evidence that antibody with more than one specificity in the same molecule is formed?

Dr. Boyd: The antibodies could be absorbed out separately but that does not prove there were no cross reactions.

Dr. Heidelberger: I think anticarbohydrate must behave differently from antiprotein. Even after immunization with four separate pneumococcal type-specific polysaccharides, subjects still responded just as well when injected with two new types.

Chapter 6

THE UNITY AND DIVERSITY OF ANTIBODIES[1]

By Elvin A. Kabat, *College of Physicians and Surgeons, Columbia University*

The Unitarian Theory of antibodies developed in the formative period of the science of immunology, when lack of knowledge of the degree of complexity of mixtures of antigens such as bacteria had led many investigators to believe that agglutinin was a substance or property of a serum different from precipitin, protective antibody, or complement-fixing antibody. In those days it required merely the appeal to "self-evident truth" to prove that agglutinin and precipitin were different entities. After all one had only to take an antiserum and it would fail to precipitate with a bacterial extract when diluted 5 to 10 times, but that same antiserum might be diluted several thousand times and yet agglutinate intact bacteria. By such an argument the burden of proof fell upon those who would contend that agglutination and precipitation were properties of one and the same substance in an antiserum—namely antibody—provided that the system involved only antibody to a single antigen.

The Unitarians readily disproved both theoretically and experimentally the conclusion drawn from the disparity in titer at which precipitation and agglutination took place. Zinsser (1) computed that much less antibody would be required to cause agglutination than precipitation and Jones (2) and others (3) showed that collodion particles coated with agg albumin could be agglutinated at a much higher dilution of anti-egg albumin than that at which precipitation could be observed. Other data from chemical fractionation also showed that the various manifestations of antibody activity were closely associated and could not be separated (4). With the development of the quantitative precipitin and agglutinin methods in the laboratory of Dr. Michael Heidelberger (5, 6), it was possible to demonstrate on a weight basis

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the identity of the agglutinin and precipitin to the type specific polysaccharides of pneumococcus (7). After this date objections to the Unitarian Theory as formulated by the early immunologists seem to have disappeared.

The same quantitative immunochemical methods, however, while demonstrating beyond reasonable doubt the validity of the Unitarian theory in the form in which it was originally conceived immediately provided evidence for the inhomogeneity or diversity of antibodies—evidence which has been amply corroborated and supplemented by physicochemical methods such as ultracentrifugation and electrophoresis (6). This heterogeneity even of antibodies to a single antigen had long been recognized in the form of cross reactions (8). For instance, crystalline duck egg albumin precipitates but a portion of the antibody from an antiserum to crystalline hen egg albumin (9). It would follow, therefore, that there is in the serum a diversity of the antibodies to crystalline hen egg albumin—some of the molecules are antibodies to both hen and duck egg albumin and others are only antibody to hen egg albumin. Additional evidence for the diversity of antibody to a single antigen was provided by Heidelberger and Kendall (10) who showed that methylation of S III yielded a product which could only remove a portion of the antibody from Type III antipneumococcal serum, the remaining antibody being removed from the supernatant only by the homologous S III.

From a quantitative study of the cross reaction of Types III and VIII pneumococcus polysaccharides (11, 12), it was demonstrated that even the cross-reacting portion of the antibody was not homogeneous but consisted of a mixture of antibodies of different reactivity toward the cross-reacting antigen. Oddly enough, the equations of the precipitin reactions (11, 12) calculated to 1.00 mg. antibody N/ml. of a given serum before and after absorption of the cross-reacting antibody were the same, indicating that the distribution of cross-reacting groups on a portion of the antibody was accomplished in a manner such as not to affect their reactivity with homologous antigen. How this is accomplished remains a crucial unsolved problem in the relation of chemical structure to immunological specificity.

Another line of evidence indicating the existence of antibodies of different reactivity to a single antigen came from the experiments on the serial absorption of antisera. With crystalline egg albumin and

crystalline horse serum albumin, the existence of two forms of antibody —precipitable and non-precipitable (or in terms of the framework theory of precipitation "multivalent" and "univalent" antibody)—was demonstrated (13, 14). The existence of the two types of antibody in varying proportions in different antisera or at different times in the serum of a given animal explain satisfactorily many interesting immunochemical findings. For instance, changes in the precipitin reaction equations per mg. antibody N during immunization in individual animals are readily explainable in terms of an increase in the number of antibody groupings per molecule (13). The finding of non-precipitable anti-egg albumin in the early bleedings of a horse immunized with egg albumin (15, 16) and later studies establishing that non-precipitable antibody to rabbit globulin was produced in the horse by subcutaneous injection while antibody of the precipitin type resulted from intravenous injection (17) are also explained. The problem is further complicated in the horse since this species may produce two types of precipitable antibody—one giving the usual precipitin type of reaction and the other giving the flocculation or so-called toxin-antitoxin type of reaction—the latter type resulting only from subcutaneous injection of certain antigens (15, 18). It has, however, not been shown that precipitating and flocculating types of antibody have ever been produced in the horse to the same antigen, so that the present distinction between precipitating antibodies (regardless of type) and non-precipitating antibodies is not ambiguous. Differences in the biological effects of these two antibodies exist. For instance, the capacity to produce passive anaphylaxis in guinea pigs of the non-precipitable antibody and the mixture of antibodies in whole serum per unit weight is equal (19), while the non-precipitating antibody is relatively ineffective in producing passive Arthus reactions (20). The capacity of rabbit antiovalbumin to sensitize guinea pig skin has been stated by Sherman, Menzel, and Seebohm (21) to reside in the non-precipitable antibody.

Antibodies to a given antigen manifest diversity in other ways which have not yet been correlated with differences in reactivity. Antipneumococcal horse antibodies may show differences in molecular weight and in electrophoretic mobility (6). Similar differences have been noted in electrophoretic mobility among antitoxins. With both these types of horse antisera these differences appear to be related to the duration of immunization. Prolonged immunization caused the appearance in

horse antipneumococcal sera of antibodies of lower molecular weight and lower electrophoretic mobility than were present in the serum of early bleedings from the same horses (22, 23, 24). Similar changes have also been reported in electrophoretic patterns of a horse under immunization with tetanal antitoxin, except that the antibody of lower mobility appeared first (25). Studies of various investigators with diphtheria antitoxin have shown that the combining proportions of these two antitoxins with different electrophoretic mobilities were not the same (26, 27). Syphilitic human sera have yielded purified Wassermann antibody with sedimentation constants of 7 and 18 (28). Goodner and Horsfall have obtained fractions of antipneumococcal antibody which varied their protective power per milligram antibody nitrogen (29).

The above group of instances, in the writer's opinion, represent the best-established instances of the diversity of antibodies. In two other fields differences in the behavior of antibodies under varying conditions have been interpreted as due to differences in the valence or combining capacity of antibodies. These are in certain human allergies and among the Rh antibodies.

In the field of allergy, Cooke (30, 31), Harley (32), Loveless (33), and others (34) have demonstrated the existence of two different kinds of antibody. One of these antibodies is found in the serum of untreated individuals sensitive to ragweed pollen and possesses the capacity to induce passive sensitization of the wheal and erythema type; it is termed skin-sensitizing antibody. The other is induced either in sensitive individuals or in normal individuals by parenteral administration of ragweed extracts. It is termed blocking antibody, since it can combine with antigen to prevent it from reacting with skin-sensitizing antibody. Miller and Campbell (35) have reported removal of skin-sensitizing antibodies from serum of humans sensitive to eggs by absorption on specific precipitates of egg albumin and rabbit anti-egg albumin and have claimed that this establishes the "univalent" character of skin-sensitizing antibody. Unfortunately, the data for the amount of skin-sensitizing antibody present in their human sera corresponds closely to the complement content of the sera—and no attempt was made by these workers to remove complement. Furthermore, if the combining proportions of skin-sensitizing antibody for antigen are taken into account in relation to the weight of skin-sensitizing antibody alleged by Miller and Campbell to be present, each antibody molecule can be computed to have to com-

bine with from 40 to 100 molecules of antigen and hence would have the unusual valence not of one but of about 40 to 100. The writer and Dr. Mary Loveless carried out such experiments in 1940 and decided therefore not to publish them. At present, while there seems to be little doubt that these two types of antibodies exist, there is no valid evidence to support the interpretation that they are due to differences in combining capacity for antigen. One possibility which deserves consideration is that the difference may not be in the antibody properties, but in some spatial configuration of the two molecules which permits one of them to attach itself to skin. In this field especially the use of purified single antigens will probably be essential to rapid progress. The data of Bukantz, Johnson, and Hampton (36) indicating that blocking antibody will reduce the amount of specific precipitate formed by ragweed extracts and rabbit anti-ragweed deserves further study, although unfortunately it does not appear to lend itself readily to quantitative measurements unless conditions are found under which the blocking antibody could be gotten to add on to specific precipitates.

While it is now evident that diversity or heterogeneity of antibodies is not too difficult to establish, each new instance of such heterogeneity should be critically examined to be sure that the evidence is not based on reasoning as fallacious as that originally cited to prove that agglutinin and precipitin were different substances. One such instance has recently appeared in the blood-grouping field in which the existence of a so-called "third order" of anti-Rh antibodies—cryptagglutinoids—has been suggested (37, 38, 39). These antibodies purportedly combine with antigen to coat red cells, but do not themselves agglutinate or block agglutination of the erythrocytes. The coating of the red cells is detected by the use of rabbit antibody to human gamma globulin—the so-called Coombs test (40). The fallacy in this approach is the failure to recognize that the Coombs test is one of the most sensitive tests for antibody, and it can readily be demonstrated that cells exposed to dilutions of anti-Rh antibody which no longer agglutinate in saline or in albumin can be shown to give a positive Coombs test. Therefore, it appears unnecessary to postulate the existence of this third form of antibody.

The existence of two types of anti-Rh antibodies, one of which agglutinates in saline and the other of which requires a high protein concentration, is also well established. However, the varied inferences drawn as to the valence of the saline agglutinin and blocking antibodies (41),

as they are called, involve a multiplicity of gratuitous assumptions. Critical scrutiny of the available evidence indicates that the crucial difference between these two types of antibody lies in the respective availability to them of groupings on the surface of the red cell. Two observations bear especially on this point. First, treatment of the red cells with trypsin causes them to be agglutinated in saline suspension by the blocking or incomplete type of antibody (42, 43), and, if the framework theory of antigen-antibody combination is accepted, these antibodies must have at least two groupings on their surface. Secondly, and even more significant is the finding of Race, Sanger, and Selwyn (44) of a family of the genetic composition —D—/—D—; in which four of the Rh-Hr factors, C, c, E, e, and their variants were lacking, and in which anti-C and anti-c and anti-e were present in the serum of one of these individuals. Erythrocytes of this genetic composition were agglutinated by blocking and incomplete anti-D in saline, again indicating, in line with the framework theory, that it cannot be univalent. Sturgeon (45), however, has recently reported that this effect was not maximally obtainable with all anti-Rh blocking sera. It is of especial interest to point out that, were all human erythrocytes of the genetic composition —D—/—D—, the two types of anti-Rh could not have been recognized, at least in certain antisera (see 45). It may be that the difference between these two types of antibody depends upon their specificity for different portions of the Rh molecule. In this field as well, progress will be largely dependent upon the preparation of purified antigens so that the methods of quantitative immunochemistry may be applied.

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Chapter 7

VALENCE OF ANTIBODIES

By A. M. Pappenheimer, Jr., *Department of Microbiology, New York University College of Medicine*

In their development of the theory of the precipitin reaction, Heidelberger and Kendall (1), Marrack (2), and later Pauling (3) and others (4, 5) found it necessary to assume that both antigen and antibody contain multiple specific combining sites. In other words antigen and antibody are "multivalent" with respect to one another. According to what has been termed the "framework" or "lattice" theory, not only is the primary union between antigen and antibody a specific one, but the subsequent aggregation of the primary complex eventually leading to precipitation must also be specific. It cannot be denied that non-specific factors such as salt concentration and lipids may accelerate or hinder this specific aggregation.

There is a great deal of evidence that antigen molecules can combine with many molecules of antibody. In general, the larger an antigen molecule, the more specific determinant groupings it contains and the more molecules of antibody it can bind. I do not believe that anyone today seriously doubts the multivalence of antigen with respect to antibody, and we may regard the problem as settled. Experimental evidence for the presence of more than one combining site on the antibody molecule is less abundant, though in my personal opinion it is no less convincing. It is concerning this question of multivalence of antibody that most of its opponents have attacked the lattice or framework theory of the precipitin reaction. There are still those who feel that multivalence of antibody has not been demonstrated satisfactorily and that aggregation of the initial specific antigen-antibody complex is governed primarily by non-specific physicochemical factors (6, 7).

We are particularly interested in the problem of "valence" of antibody in this symposium because a good deal of evidence has been accumulating which indicates that the kind of antibody concerned in certain types of allergic reactions in man differs in reactivity from ordinary precipitating antibody. It has been said that skin-sensitizing antibodies,

or "reagins" as the allergists call them, may represent antibody which is "univalent" and thus contains only a single specific combining site. Evidence in favor of this hypothesis will be presented in some of the papers which follow. Moreover, "univalent" antibodies have recently been postulated to explain peculiarities in other types of immunological reactions. Thus, on rather slender evidence, the so-called "blocking" antibodies encountered in certain anti-Rh sera are regarded by some as being of the "univalent" type.

Even at the risk of reopening an old and perhaps worn-out controversy, it seems worthwhile to review some of the evidence concerning the valence of precipitating antibody. Several examples of experimental evidence which demonstrate that antibody may possess more than one combining site per molecule will be presented.

Let us first consider the behavior of a particular antigen-antibody system in the inhibition zone of antigen excess (8). In Figure 1 is shown the sedimentation diagram of a highly purified horse γ_1-globulin prep-

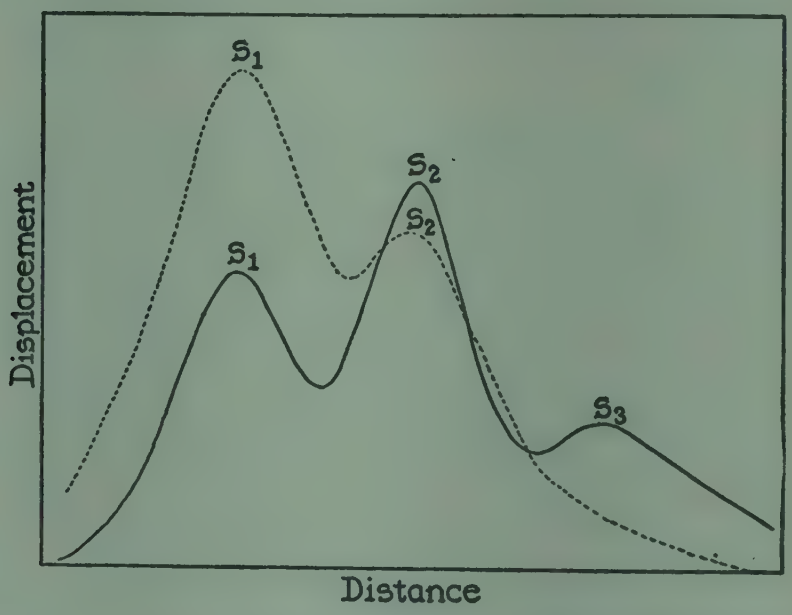


FIGURE 1. SEDIMENTATION DIAGRAM OF MIXTURE OF TOXIN AND ANTITOXIC PSEUDOGLOBULIN IN REGION OF TOXIN EXCESS
Heavy line: 0.965 mg. toxin nitrogen (2100 Lf per ml.) and 1.135 mg. pseudoglobulin nitrogen per ml. (275 units per ml.) of which 0.637 mg. is inactive pseudoglobulin. Broken line: 0.965 mg. toxin nitrogen per ml. and 0.500 mg. inactive toxin-absorbed pseudoglobulin nitrogen per ml. (From *J. Exp. Med.*, 1940, 71:259.)

aration of known diphtheria antitoxin content after having been allowed to come to equilibrium with a large excess of highly purified diphtheria toxin. The solid line in Figure 1 shows three components: S_1, uncombined toxin of sedimentation constant 4.5S, S_2, inactive γ_1-globulin and a small amount of S_3 with a sedimentation constant of about 9S which presumably represents an unaggregated complex of toxin with antitoxin probably of composition TA. All the remaining toxin and antitoxin had combined to form a large aggregate which had already sedimented to the bottom of the cell at the time the photograph was taken from which this diagram was made. The broken curve in Figure 1 represents a control experiment in which the same amount of toxin was sedimented with an equivalent amount of inactive non-antitoxic γ_1-globulin. Since the areas under each component are proportional to their concentrations, we may calculate the amount of toxin specifically bound to antitoxin by simply subtracting the area under component S_1 of the lower curve from the area under the corresponding S_1 component of the control curve. The antitoxin content of the serum is known, as are the molecular weights of both toxin and antitoxin, so that the molecular ratio of combined toxin to antitoxin may be readily calculated. This has been done in three such experiments and from the table we can see that 1.5 or more molecules of toxin are combined for each molecule of anti-

SEDIMENTATION OF DIPHTHERIA ANTITOXIC HORSE GAMMA$_1$-GLOBULIN IN EXCESS PURIFIED TOXIN

Toxin Added	*Combined Toxin*	*Combined Antitoxin*	*Molecular Ratio*[a] *Toxin/Antitoxin*
mgs./c.c.	mgs./c.c.	mgs./c.c.	
6.04	2.00	3.22	1.5
3.33	2.81	3.22	2.1
4.06[b]	1.93	2.76	1.6

[a] Assuming molecular weight toxin = 70,000, antitoxic gamma$_1$-globulin = 165,000.

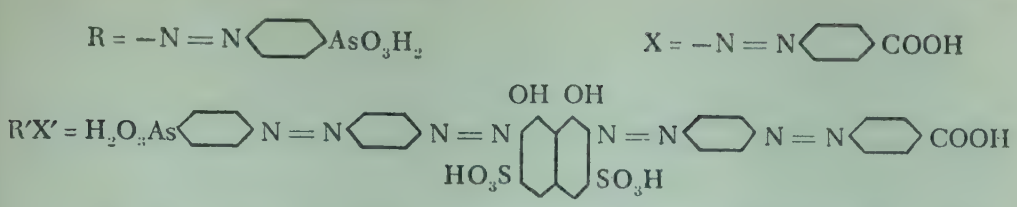
[b] Toxin-antitoxin floccules dissolved in excess toxin.

toxin present. The last experiment shown in the table was carried out using toxin-antitoxin floccules dissolved in excess purified toxin. In this case only two components were observed in the ultracentrifuge, since no non-specific γ-globulin was present. It is thus evident that the same equilibrium may be reached from both sides. Recently, Marrack *et al.* (9) have carried out similar experiments on rabbit antialbumin-albumin

specific precipitates dissolved in albumin excess using electrophoresis for separating the various components. Marrack also found ratios of antigen to antibody that were greater than one for both horse serum albumin and ovalbumin systems. It is difficult to see how these experiments can be interpreted in any other way save to indicate that most of the antibody present in the sera was at least bivalent.

A quite different type of experiment recently reported by Eisen and Karush (10) provides additional evidence for the multivalence of antibody. Rabbit antibody, the specificity of which was directed against p-azo-phenyl-arsonic acid, was precipitated with erythrocyte stromata coupled with diazotized p-arsanilic acid. Highly purified antibody was obtained by dissociation and elution of the precipitate at pH 3.8. This purified antibody was then used in equilibrium dialysis experiments against the homologous haptenic dye, p-(p-hydroxyphenylazo)-phenyl-arsonic acid. The amount of dye bound by antibody was measured at different dye concentrations. By extrapolation of the curve obtained by plotting the reciprocal of moles hapten bound per mole of antibody against the reciprocal of the free hapten concentration, a value of 2 for the average binding capacity of antibody was obtained. It is obvious that in these experiments no aggregation could occur (according to the framework theory) since the hapten possessed only one determinant group.

Pauling, Pressman, and Campbell (11) have collected a considerable amount of data using synthetic haptenes, the immunological behavior of which is most readily explained in terms of the framework theory of the precipitin reaction. To illustrate the type of evidence Pauling and his co-workers have provided, let us consider an ingenious set of experiments which they have reported on the precipitation of two specific antisera by a dihaptenic substance containing one of each of the two corresponding determinant groups. Pauling *et al.* prepared azoprotein antigen containing R groups (p-azophenylarsonic acid) and azoprotein antigen containing X groups (p-azobenzoic acid) and immunized rabbits to obtain specific antisera directed against each of these haptenes. It was then found that the synthetic hapten R′X′ (shown below), which contains one R-grouping and one X-grouping, failed to precipitate either anti-R or anti-X serum alone, but gave good precipitation with a mixture of the two antisera.



If r = anti-R and x = anti-X and each antibody possesses two specific combining sites, then the precipitate formed when R′X′ is added to a mixture of the two antisera should have the following composition:

$$(-\,r\,-\,R'X'\,-\,x\,-\,X'R'\,-\,r\,-\,R'X'\,-\,x\,-\,X'R'\,-)_n = \left\{(R'X')_2 rx\right\}_n$$

Using quantitative methods it was shown that at the point of optimal precipitation the antigen-antibody molecular ratio in the specific precipitate was 0.7, indicating an average valence of 2.8 for antibody.

While the haptenic substance R′X′ failed to precipitate anti-R or anti-X sera separately, needless to say each antiserum could be precipitated by homologous haptenes of the type R′R′ and X′X′. Additional evidence supporting the multivalence of antibody is suggested by the fact that precipitation of the mixture of antisera could be inhibited by either of the haptenes R* or X*[1] as well as by excess antibody.

The experiments of Pauling *et al.* have been criticized on the grounds that the haptenes employed form aggregates (Pardee and Swingle, 12) and therefore contain multiple determinant groups (Haurowitz, 7). It has also been pointed out by Kabat (13) that the conditions used by the Pauling group did not allow sufficient time for complete precipitation to take place.

Let us finally consider some evidence which suggests that in the case of equine antipolysaccharide antibody of high molecular weight the number of specific combining sites per antibody molecule may be much greater than 2 and such antibody may possess a valence of 10 or 20 or even more. Antipneumococcal antibody in the horse is generally associated with an assymetric γ-globulin component of molecular weight approximately 1,000,000 (14). If such antibody is treated with pepsin, it may be depolymerized and precipitating antibody units of molecular weight less than 100,000 (15) may be recovered in good yield. Thus even if the reactive fragments are presumed to be univalent, the original

[1] R* = p-(p-hydroxyphenylazo) phenylarsonic acid. X* = p-(p-hydroxyphenylazo) benzoic acid.

antibody in its polymerized form must have possessed 5 to 6 or more specific combining sites. Some years ago Dr. Mary Petermann, then at the University of Wisconsin, and I studied the behavior of a Type II antipneumococcal serum in some detail. This serum (513–50) was obtained from a horse which had been under immunization for several years. Examination of the euglobulin fraction showed a high degree of inhomogeneity with most of the antibody concentrated in high molecular weight components. Purification of the antibody was accomplished by differential centrifugation of the euglobulin fraction. After 4 hours' sedimentation in a Beams air-driven centrifuge at 25,000 r.p.m., virtually all the antibody could be recovered as a thick syrup from the bottom of the centrifuge tubes. The sediment on resolution showed two major components when examined in the analytical ultracentrifuge with sedimentation constants 17.4S and 29.8S, both of which were of high antibody content. This antibody of high molecular weight, prepared by concentration in the Beams centrifuge, was then subjected to peptic digestion at pH 4.5 and 0° C. following the procedure of Grabar (16). In agreement with Grabar and with Treffers and Heidelberger (17), it was found that the enzyme-treated pneumococcal antibody was capable of combining with twice as much Type II polysaccharide per milligram antibody nitrogen as the original material before treatment with pepsin. Moreover, it was found that, provided digestion proceeded for a sufficient length of time, all of the digested antibody showed a sedimentation constant of only 5.2S, suggesting a molecular weight of less than 100,000 as compared to an original molecular weight of nearly 1,000,000 for the 17.4S component. From these findings we may infer that high molecular weight equine antibody is built up of 5 or 6 units of molecular weight similar to that usually found for γ-globulin (165,000) polymerized end to end. On treatment with pepsin the large assymetrical antibody molecule is first depolymerized to these smaller units, each of which is then further split into two fragments, one of which combines with twice as much S II per unit weight as the original antibody. The other fragment is completely inactive. In support of this picture there is definite evidence that if peptic digestion is not carried on long enough, the digestion mixture contains an appreciable proportion of antibody with sedimentation constant of 7S, or that of normal globulin. The work of Kabat (18) also suggests that high molecular weight antibody may be built up by end-to-end polymerization of smaller units. Dissociation

of specific precipitates with barium hydroxide at alkaline pH results in depolymerization to smaller units without change in specific activity (18). The structure of high molecular weight equine Type II antipneumococcal antibody is shown diagrammatically in Figure 2.

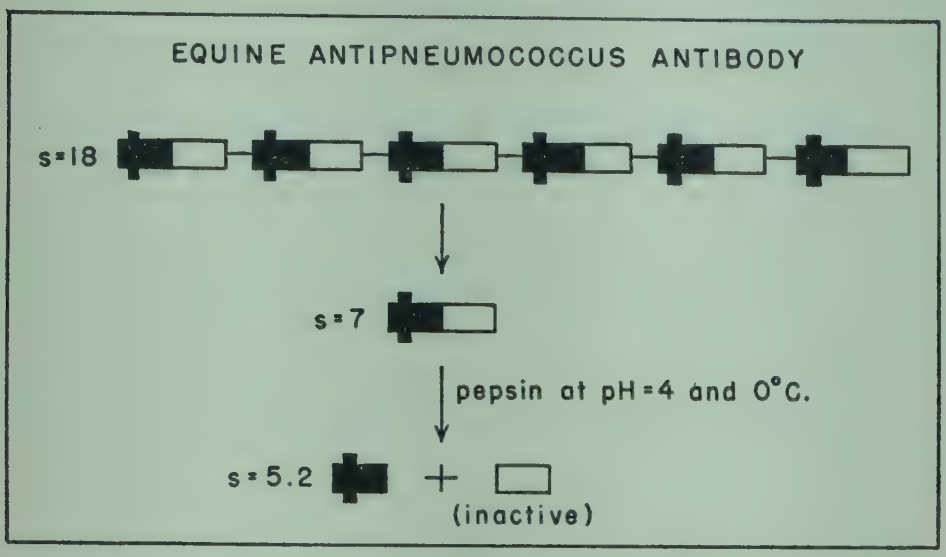


FIGURE 2. DIAGRAM REPRESENTING STRUCTURE OF HIGH MOLECULAR WEIGHT EQUINE ANTIPNEUMOCOCCAL ANTIBODY

It was also found that following peptic digestion of the Type II antibody a further separation into two antibodies of different reactivities, one water soluble, the other insoluble, could be obtained by simple dialysis. The sedimentation constant of both fractions was 5.2S. The quantitative immunochemical behavior of these two fractions together with that of the original undigested antibody is shown in Figure 3; the ratio of milligrams antibody nitrogen to milligrams of antigen in the specific precipitate is plotted as ordinate against antigen added as abscissa. It can be seen that both digested fractions combine with more antigen per unit weight than the original antiserum. Moreover, the water soluble antibody combines with twice as much antibody as the water insoluble fraction, despite the fact that both fractions have the same sedimentation constant. In fact the water soluble material combines with so much polysaccharide that even precipitates formed in the region of antibody excess are gummy, hard to break up, and resemble those usually formed only with an excess of antigen. Thus it is possible that the water soluble antibody possesses twice as many combining sites per

molecule as the water insoluble material. If it is assumed that precipitating antibody must be at least bivalent and that the difference between the above two antibody fractions may be attributed to a difference in valence, then the original undigested antibody of high molecular weight must have consisted of a mixture of molecules of valence 10-12 and 20-24 or even more.

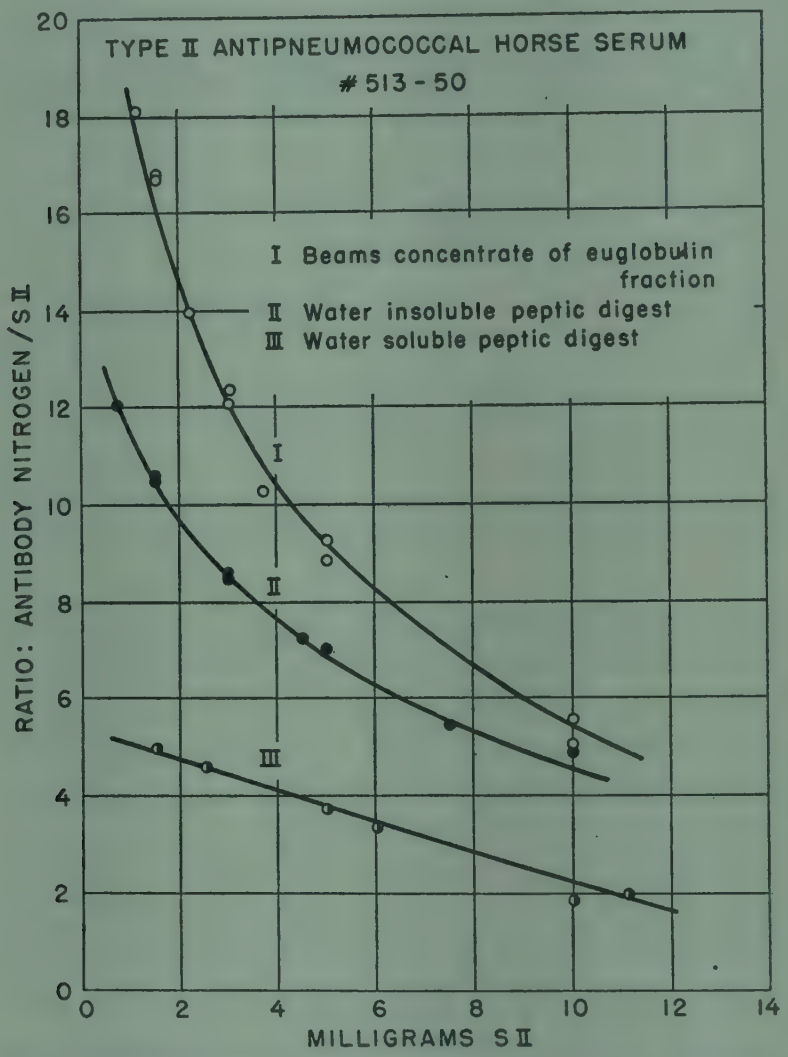


FIGURE 3. QUANTITATIVE PRECIPITATION OF VARIOUS GLOBULIN FRACTIONS FROM TYPE II ANTIPNEUMOCOCCAL HORSE SERUM NO. 513–50

This interpretation is admittedly open to question and one would be quite justified in asserting that the difference in reactivity of the two antibodies could be ascribed equally well to differences in their specificity, as has been shown to be the case with antibodies directed against

Types III and VIII polysaccharides (19). Type II antibody cross reacts with Type V pneumococcal polysaccharide. It is known from previous studies (20) that Type II antiserum contains two antibodies of different specificity, one of which is completely precipitated by either Type II or Type V polysaccharides, the other is specific for Type II only. It is possible that, as with Types III and VIII, we are dealing here with two antibodies, one of which is directed against a repeating unit consisting of twice as many hexose and uronic acid groupings as the other. Unfortunately, it was not possible to test this possibility at the time the above experiments were carried out.

It may be added that the ultracentrifugal as well as the immunochemical studies on these Type II antibody fractions were repeated using salt-dissociated antibody of high immunological purity. The dissociated antibody showed the same quantitative immunological activity as the original globulin fractions from which the specific precipitates were prepared.

NON-PRECIPITATING ANTIBODIES

Clearly, if the framework theory is correct, no specific precipitation will occur if *all* the antibody present in a serum contains only a single combining site or valence per molecule. Providing that some bivalent or multivalent antibody is present, however, there is no reason why "univalent" antibody should not be specifically coprecipitated by adding on to the free combining sites of antigen in a specific aggregate or framework. On the other hand, it should be emphasized, at the very outset, that failure of an antibody to be precipitated does not necessarily mean "univalence" even if the antibody is known to be present in significant concentration.

It was shown some years ago by Heidelberger and Kendall (21) that when rabbit anti-ovalbumin sera were absorbed with several small charges of ovalbumin, an appreciable amount of antibody frequently remained in the supernatant which failed to precipitate on further addition of ovalbumin. Such "incomplete" or "univalent" antibody, as it has been called, can be carried down quantitatively on the specific precipitate formed when more precipitating anti-ovalbumin and a suitable amount of ovalbumin are added. The proportion of such non-precipitating or univalent antibody varied in different sera which they

studied but seemed to be present in greatest amount in the serum of animals which had been under immunization for a long time.

Prolonged immunization is not always necessary for the production of non-precipitating antibody, however. Thus an early bleeding from a horse immunized with ovalbumin showed no precipitating or flocculating antibody whatever, but was capable, nevertheless, of inhibiting the precipitation of ovalbumin by rabbit anti-ovalbumin (22). Heidelberger, Treffers, and Mayer (23) showed that when precipitation of ovalbumin-rabbit anti-ovalbumin finally did occur, more than 1 mg. of "univalent" equine antiovalbumin could be carried down per c.c. of serum.

There are a number of means by which precipitating antibody may be artificially converted in the laboratory to a non-precipitating form with properties compatible with univalence. Thus treatment of various antisera with diazo-compounds (24), formaldehyde (25), and by photo-oxidation (26) has resulted in loss of precipitability without concomitant loss in ability to combine with or neutralize the homologous antigen. In most of these studies, however, the proportion of antibody completely destroyed by the treatment is not known, since quantitative co-precipitation experiments of the type discussed above were not carried out. Kleczkowski (27) has shown that rabbit antibodies may be converted to a non-precipitating form when heated to 70° C. in the presence of albumin and that loss of precipitability of such heated antibody is due to complex formation with serum albumin. In the case of human, guinea pig, and monkey diphtheria antitoxin, conversion to a non-precipitating form takes place at more moderate temperatures (56° C.) in the presence of serum albumin. Heated human antitoxin retains at least 90 percent of its capacity to neutralize diphtheria toxin and may be quantitatively recovered by co-precipitation with rabbit antitoxin and toxin (28). Although complex formation with serum albumin, as in Kleczkowski's experiments, was not demonstrated, it was found that *purified* human antitoxic γ_2-globulin is unaffected by prolonged treatment at 56° C. Addition of a crude albumin fraction to the purified γ_2-globulin restored the original lability. It is interesting to note that high titers of non-precipitating antitoxin have been observed in the *unheated* serum of certain allergic patients hyperimmunized with purified diphtheria toxoid. Kuhns and Pappenheimer (29) have studied several such indi-

viduals who showed hypersensitivity of the immediate type to diphtheria toxoid and whose sera contained as much as 100 units (1.5 mgs.)[2] non-precipitating antitoxin per ml. which could be co-precipitated with rabbit antitoxin.

There is another method by which the precipitability of an antiserum may be affected. Many years ago Hartley (30) showed that removal of lipids from diphtheria antitoxin by extraction with alcohol and ether at low temperature resulted in a loss in flocculating power without, however, altering the *in vivo* neutralizing titer. Hartley found that the precipitability of a number of different extracted antisera was similarly affected. Later, Horsfall and Goodner (31) showed that extraction of equine and rabbit anti-pneumococcal serum by the Hartley procedure resulted in an analogous loss in visible agglutinating and precipitating power. They succeeded in restoring the initial activity of horse serum by addition of lecithin and of rabbit serum by addition of cephalin. Recently Krueger and Heidelberger (32) have extracted the lipids from equine anti-rabbit serum albumin and anti-globulin sera and have concluded that the effect of lipid removal is mainly on *rate* of aggregation. By centrifugation at sufficiently high speed of the complex formed between lipid-extracted antibody and homologous antigen which had been allowed to interact 5–10 days or even longer, the same amount of specific precipitate may be recovered as from low-speed centrifugation of unextracted antigen-antibody complex.

Recent work reported by Katsura (33) is of particular interest in connection with the role of lipids. Katsura repeated Hartley's original experiments with lipid-extracted equine diphtheria antitoxin. By blending with a rapidly flocculating antitoxin he was able to show that extracted antitoxin could be quantitatively recovered by co-precipitation. Katsura was not able to restore flocculating power by addition of lecithin. The particular significance of Katsura's work lies in his finding that purified antitoxic pseudoglobulin is only slightly affected by lipid extraction unless mixed with normal serum or certain serum fractions. Similarly, the flocculation time of purified pepsin-treated antitoxin is unaltered by lipid extraction. Moreover, when non-flocculating lipid-extracted antitoxin is subjected to partial digestion with pepsin, its flocculating power with toxin is completely restored. Finally, some evidence was obtained

[2] Calculated by assuming 15 μgs. antitoxic globulin per unit (28).

that lipid extraction involves changes in certain electrophoretic components including that associated with the antitoxin. Katsura's experiments suggest that removal of lipids from antitoxic sera may permit interaction of some non-specific protein component with antitoxin so as to interfere with one of its toxin-binding sites and thus effectively hinder aggregation.

How can we be assured that antibody which fails to precipitate with homologous antigen is truly univalent? Clearly mere failure to precipitate or agglutinate constitutes insufficient evidence. In the case of the so-called "blocking" type of Rh antibody (34), for example, it can readily be demonstrated that combination with red cells takes place even though the cells are not agglutinated. However, treatment of the cells with trypsin (35) or suspension in serum albumin (36) renders them agglutinable by the same "blocking" antibody. Obviously, the failure of "blocking" antibody to agglutinate untreated cells cannot be attributed to its univalence.

At the present time there appears to be no method available for proving the existence of antibody with only a single determinant site. Failure to aggregate in the presence of multivalent antigen together with the capacity to add specifically to an homologous precipitating system constitutes evidence that only one combining site is active. However, in such cases it is always possible that interaction has taken place of the type implied by Kleczkowski's experiments with heated antisera (27) and Katsura's (33) using lipid extraction. In such instances the possibility always exists that by suitable treatment the blocked combining sites may be uncovered and multivalent precipitating antibody regenerated.

In conclusion it would appear to me that the evidence for multivalence of antibody is sufficiently convincing so that it need no longer be considered the problem of primary concern. In my opinion, the subject of particular current interest is the nature of the naturally occurring or artificially produced non-precipitating antibodies which have been loosely termed "univalent." Are the skin-sensitizing reagins found in allergic patients truly univalent or is some other mechanism involved? Is it possible that failure of such antibodies to form precipitable aggregates with antigen is related to some alteration in lipid content or to masking of a specific combining site by interaction with some non-specific serum protein?

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DISCUSSION

Dr. A. S. Wiener: In regard to the valency of Rh antibodies, univalent Rh antibodies (or "blockers") are capable of crossing the placental barrier, while bivalent antibodies (or saline agglutinins) do not cross the placenta.

The agglutinability of Rh-positive red cells by univalent antibodies in suspension media such as a mixture of serum or plasma and albumin is of interest. The univalent Rh antibodies coat Rh-positive red cells in a saline medium although clumping does not occur. When a plasma-albumin mixture is substituted for saline, conglutinin is provided. This produces a condition of "stickiness" on the coated cells which consequently form aggregates.

Our studies on exchange transfusion have given us some opportunity to observe antibody response and the behavior of antibodies in the newborn. If a newborn infant of blood group O with a group B mother is exchange transfused with B blood, one observes a linear curve of red cell destruction and some type B cells are still found at four months. Yet no detectable beta antibodies are found then or even up to six months. This illustrates the poor types of antibody response found in infants.

Dr. Haurowitz: I still do not feel that the evidence in favor of bivalence of rabbit antibody is convincing. On the contrary, much of the evidence is more readily interpreted if we assume that antibody has only one specific reacting site. This is supported by the following facts: (*1*) Rothen and Landsteiner have shown that antibody is specifically adsorbed to layers of the antigen, but that layers of antibody are not able to adsorb antigen. (2) Enzymatic digestion results in splitting the horse antitoxin molecule in two, yielding an inactive fragment and one with nearly twice the specific activity of the original molecule. If antitoxin is multivalent, why are not both fragments antitoxic? (3) When rabbits are immunized with bovine serum globulin, both antigen and antibody are gamma globulins. Yet the specific precipitate is only soluble in excess bovine gamma globulin, not in excess rabbit globulin. This is most readily explained in terms of the univalent theory. Precipitation has nothing to do with valence. Solubility depends on other factors.

Dr. Heidelberger: With respect to Dr. Haurowitz's example where both antigen and antibody are gamma globulin, I fail to see why this system should behave differently from any other, because in the equivalence region there are three molecules of antibody globulin to one of antigen, and it is only in the region of large antigen excess that one would have a large extended molecule that would be soluble.

Dr. Pappenheimer: I think we should be cautious in interpreting results of experiments with monolayers and surface films. Unless antibodies are highly purified one may get non-specific interactions. Using purified antibodies Porter and I were able to build several alternate layers of type specific pneumococcal polysaccharides and their homologous antibodies. On the other hand a layer of horse antitoxin failed to absorb toxin, although in the

reverse case a monolayer of toxin was capable of specifically absorbing antitoxin. No further layering of toxin was possible in the latter instance. Our own interpretation of the monolayer experiments with the toxin-antitoxin system and of the action of proteolytic enzymes on horse antitoxin is that the results suggest that the specific reactive sites lie sufficiently close together on the antitoxin molecule so that they may interact. Kendall has shown mathematically that such interaction will account for the flocculation type of reaction with the soluble zone in antibody excess observed in the equine toxin-antitoxin system.

Chapter 8

EXPERIMENTAL PRODUCTION OF SKIN-SENSITIZING ANTIBODIES

By William B. Sherman, *College of Physicians and Surgeons, Columbia University, Roosevelt Hospital, and Presbyterian Hospital*

In early studies of the Prausnitz-Küstner reaction, Coca and Grove (1) attributed the skin-sensitizing activity of sera from patients with spontaneously developed allergies to the presence of the *atopic reagin,* which they believed to be peculiar to the human species and to have been developed as a result of hereditary predisposition. They reported that rabbit anti-egg white and anti-ragweed precipitating sera did not passively sensitize human skin, that the reagin lacked precipitation activity, failed to passively sensitize guinea pigs, and also differed from the anaphylactic antibody in being relatively labile at 56° C. Subsequent studies have shown that skin-sensitizing antibodies indistinguishable from typical reagins are formed by man during serum sickness (2), regardless of the presence or absence of any known hereditary predisposition to allergy, and that similar antibodies may be demonstrated in dogs and other species with spontaneously acquired hay fever (3). Therefore, the simple descriptive term *skin-sensitizing antibody* seems preferable to reagin because of the connotations of hereditary background and peculiarity to the human species.

Since adequate amounts of heterologous antiserum has been shown to produce serum sickness in approximately 90 percent of persons, regardless of hereditary predisposition to allergy, it is apparent that skin-sensitizing antibodies to this antigen could be readily induced in man if desired. Brunner (4), Walzer (5) and others have experimentally produced skin-sensitizing antibodies to other antigens in man. Of the antigens used, only extracts of Ascaris produced this type of antibody in a majority of persons given subcutaneous injections. Extract of orris root produced sensitization in only one of 8 persons treated, and injections of rabbit epithelium and egg white had no effect. Several investigators have reported that repeated injections of relatively large amounts

of extracts of grass and ragweed pollens into normal humans do not stimulate the production of skin-sensitizing antibodies (6, 7, 8), although they do lead to the formation of thermostable blocking antibody similar to that developed in hay fever patients as a result of pollen antigen injections. Recently, Kuhns and Pappenheimer have shown that injections of diphtheria toxoid produce, in certain persons, a non-precipitating type of antitoxin which has most of the properties of a typical skin-sensitizing antibody. The individuals showing this phenomenon are usually those with a personal or family history of allergies of the hay fever type (26). Studies of experimental active sensitization in man are few because of the risks involved.

In guinea pigs, Landsteiner and Chase have shown that certain sera of animals actively sensitized to proteins or to simple chemical compounds produced local passive sensitization in the skin of normal animals manifested by a prompt urticarial response similar to the Prausnitz-Küstner reaction (9, 10, 11). However, in other studies of skin-sensitizing antibodies in animal sera, passive sensitization of human skin has been more widely utilized, both because of the clear-cut reactions obtained in human skin and because of a desire to demonstrate animal antibodies closely related to those of human allergic sera.

Cooke and Spain (2) reported the production in human skin of reactions identical with the typical Prausnitz-Küstner phenomenon by 3 of 6 sera from rabbits immunized with egg white. All of these sera contained precipitins in titers of 1:1000 to 1:3000, and all produced passive anaphylactic sensitization in guinea pigs. DeBesche (12) reported similar passive sensitization of human skin by 7 of 10 rabbit anti-horse sera.

On the other hand, Coca and Grove (13), Vallery-Radot (14), and Gyorgy, Moro, and Witebsky (15) reported negative results in similar experiments using rabbit anti-egg and anti-horse sera. More recent studies have amply confirmed the fact that some but not all rabbit and guinea pig antisera produce passive sensitization in human skin. The discordant results of early studies, most of which employed only a few antisera, are explained by the irregularity with which the skin-sensitizing property is present in the sera of various animals actively sensitized by most methods and also by the variations in acceptance of passive sensitization with animal sera by different apparently normal human subjects.

SKIN-SENSITIZING ANTIBODIES IN GUINEA PIGS

Caulfield, Brown, and Waters (16) in 1937 described the passive sensitization of normal human skin by antisera from two guinea pigs sensitized three months previously by subcutaneous injection of 2.5 ml. of alum-precipitated ragweed extract. The reactions in human skin obtained after passively sensitizing with these two antisera were definite and specific, but no mention was made of the proportion of positive results obtained with sera of guinea pigs sensitized by this method.

In similar experiments at The Roosevelt Hospital (17), 31 guinea pigs were each given a single intraperitoneal injection of 2 ml. of alum-precipitated ragweed or grass pollen extract, containing 0.5 mg. of protein nitrogen. Serum drawn after 30 to 85 days was tested in passive sensitization of human skin. Four of the guinea pigs were bled a second time 125 to 145 days after the sensitizing injection. At the end of the experiment, Dale tests were performed; six of the guinea pigs (whose sera were inactive in human skin) showed negative Dale reactions and so could not be considered actively sensitized. Of the other 25 guinea pigs, the sera of six passively sensitized human skin. The interval between the sensitizing injection and the bleeding appeared to be an important factor in the results obtained. None of 13 guinea pigs bled during the first two months showed the presence of skin-sensitizing antibody, while six of 12 bled after 2–4 months gave sera with skin-sensitizing activity (Table 1). All of the four sera obtained at the second bleedings after 125–145 days were active in human skin, including two from guinea pigs whose first sera (obtained 21–78 days after the sensitizing injection) gave doubtful results.

Passive sensitization of human skin was also studied with guinea pig anti-ovalbumin sera supplied by Dr. E. J. Coulson of the Allergen Research Laboratory of the Department of Agriculture. These antisera were from 19 guinea pigs sensitized with various doses of crystalline ovalbumin, in most instances with a single subcutaneous injection of 0.002 to 0.3 mg. of alum-precipitated antigen. Seven of the guinea pigs had survived a shocking dose of 0.077 to 0.59 mg. of dissolved ovalbumin intravenously. Sera were obtained four to 32 weeks after sensitization and six to 13 days after the challenging dose. Three of these sera passively sensitized human skin, two in a dilution of 1-100 and one, 1-10. All of these three sera were from guinea pigs which had survived chal-

lenging doses of antigen. The sera of the other 16 guinea pigs, including four which had survived anaphylactic shock, lacked skin-sensitizing activity even undiluted. It was apparent that skin-sensitizing antibodies to ovalbumin could be induced in guinea pigs, but only occasionally by the methods used (18).

TABLE 1

PASSIVE SENSITIZATION OF HUMAN SKIN WITH SERA TAKEN FROM GUINEA PIGS 60–145 DAYS AFTER INJECTION OF ALUM-PRECIPITATED POLLEN ANTIGENS

Guinea Pig No.	*Pollen Antigen Injected*	*Days before Bleeding*	*Sensitization of Human Skin*[a]		
16	Timothy	69	+++	+++	
17	Timothy	79	0	0	
18	Low ragweed	71	±	±	
19	Low ragweed	77	0	0	
20	Low ragweed	75	±	±	
20	Low ragweed	145	++	+	
21	Low ragweed	77	+	0	
21	Low ragweed	125	++	...	
22	Low ragweed	75	0	0	
23	Low ragweed	75	+	+	
23	Low ragweed	125	++	++	
24	Low ragweed	78	++	+	0
25	Low ragweed	71	+	++	0
25	Low ragweed	125	++	...	

Source: Sherman, Stull, and Hampton, *J. Immunol.*, 1939, 36:450–452.

[a] Multiple notations represent reactions on various test subjects.

SKIN-SENSITIZING ANTIBODIES IN RABBITS

Winkenwerder, Eagle, and Arbesman (25) studied the skin-sensitizing activity of the sera of rabbits injected with a suspension of ground ragweed pollen and with six different types of soluble extracts of the same pollen. Of the sera of ten rabbits injected both subcutaneously and intraperitoneally, nine contained skin-sensitizing antibodies. Of 19 rabbits treated intravenously, seven showed evidence of skin-sensitizing antibodies. The reactions produced in human skin by these sera were typical but relatively small, with wheals 0.3 cm. to 1.0 cm. in diameter. Striking variations were noted in the reactions of the skin of different individuals when sensitized with the same serum.

In our laboratory, antibodies capable of passively sensitizing human skin have been produced in rabbits by the injection of crystalline egg albumin, horse serum, bovine albumin and ragweed pollen extract. In one experiment (17), two rabbits were given 18 intracutaneous injections each of 0.1 ml. of horse serum during a period of two months. Sera obtained two weeks after the last injection showed precipitin titers of 1:5000 and 1:10,000; both sera readily produced passive anaphylactic sensitization of guinea pigs. One antiserum consistently produced passive sensitization of human skin. In one test subject, 0.1 ml. of a 1:1000 dilution of this antiserum produced definite sensitization. The serum of the other similarly treated rabbit showed no skin-sensitizing activity in five of the same human subjects (Table 2). Antisera of other rabbits

TABLE 2

ANTI-HORSE RABBIT SERA

Rabbit Sera	SENSITIZATION OF SKIN OF HUMAN TEST-SUBJECTS					*Dale Reaction on Passively Sensitized Guinea*	*Precipitin*
	Human Test Subjects						
Number	*A*	*B*	*C*	*D*	*E*	*Pigs*	*Titer*
51	++	++	++	++	++	+	1:10,000
85	0	0	0	0	0	+	1:5000
Normal	0	0	0	0	0		

Source: Sherman, Stull, and Hampton, *J. Immunol.*, 1939, 36:455.

which had been given large amounts of horse serum intravenously or alum-precipitated horse serum intramuscularly have shown passive sensitization of human skin demonstrable with dilutions of antiserum up to 1:100 but neither method has produced this type of antibody in all rabbits treated.

Eight rabbits were given intraperitoneal injections of alum-precipitated bovine-plasma albumin twice weekly for periods of two to four months. Attempts to demonstrate skin-sensitizing antibodies to this antigen were disappointing: sera of three rabbits showed only feeble activity in human skin; the other five gave negative results.

Sera of rabbits given two or three intramuscular injections of ragweed pollen antigen (each containing approximately 10 mg. of protein N)

adsorbed on aluminum hydroxide, in a majority of cases showed slight but definite activity in passively sensitizing human skin (19).

More extensive studies have been made of the sera of rabbits sensitized by subcutaneous injections of crystalline egg albumin (20). Fourteen rabbits were given eight to thirteen injections of alum-precipitated egg albumin and nine a corresponding series of injections of saline solution of egg albumin. The injections were given three times a week and each contained 10 mg. of albumin. Sera were obtained one week after the last injection. All of the 14 sera from rabbits receiving alum-precipitated antigen passively sensitized human skin, 10 showing activity in a dilution of 1:10 and six in a dilution of 1:100. Of the nine sera from rabbits sensitized with dissolved egg albumin, none produced a definite reaction in human skin, one gave a questionable (±) reaction when injected undiluted.[1] Other routes of injections have not been adequately studied. Of two rabbits treated intramuscularly with alum-precipitated egg albumin, one developed skin-sensitizing antibody while the other did not. Similar results were noted in two rabbits injected intraperitoneally with alum-precipitated antigen. Sera of two rabbits given intravenous injections and one injected intramuscularly with dissolved egg albumin all gave negative tests for skin-sensitizing antibody.

TECHNIQUES OF PRODUCING SKIN-SENSITIZING ANTIBODIES

It is apparent that antibodies capable of sensitizing human skin have been produced in rabbits or guinea pigs by the injection of horse serum, egg albumin, extracts of grass and ragweed pollens and, less satisfactorily, with bovine albumin. By most of the techniques employed only a fraction of the animals treated have developed this type of antibody and in many instances the titers have been low. The most consistent results followed repeated subcutaneous injections of alum-precipitated egg albumin into rabbits. Many more experiments would be required to define the optimal conditions for producing this type of antibody, but certain inferences may be drawn from the incomplete data available. It was apparent that alum-precipitated antigens were more efficacious than the solutions of the same antigens. A relatively long time was re-

[1] That dissolved egg white (not prepared as crystallized albumin) may produce skin-sensitizing antibody in rabbits was demonstrated by Cooke and Spain (2), who did not report in detail the procedure used.

quired for the development of such antibodies in guinea pigs following intraperitoneal injection of alum-precipitated pollen antigens. Pigs so treated became anaphylactically sensitized in less than 30 days, but skin-sensitizing antibodies were not detected before the 69th day and apparently increased in amount up to the 125th or 145th day. The results obtained with sera of guinea pigs sensitized with ovalbumin suggested that development of this type of antibody might be facilitated by intravenous challenging doses of soluble antigen following the initial sensitization with alum-precipitated antigen subcutaneously.

DEMONSTRATION OF SKIN-SENSITIZING ANTIBODIES

The technique of testing guinea pig or rabbit sera for sensitization of human skin is identical with that of passive transfer with human allergic serum. One-tenth milliliter of sterile antiserum is injected intracutaneously into the skin of the back or upper arm of a normal human subject. The sites of injections are marked with gentian violet or ink and tested 24 or 48 hours later by intracutaneous injection of 0.025 ml. of antigen solution containing 0.01 to 0.05 mg. of protein N per ml. In the selection of test subjects it is obviously essential to eliminate by preliminary skin tests those reacting either to the antigen or the animal serum being used. A number of apparently normal persons showing no immediate reaction to test with the heterologous serum, develop a delayed inflammatory reaction in the sensitized sites which is still present at the usual time of testing with antigen. Sites showing such irritation do not give reliable reactions to antigen and are of no value. This type of delayed reaction is believed to result from a minor degree of sensitization to the animal serum. Occasionally the test subjects develop local serum sickness manifested by urticaria at the sensitized sites. Ordinarily this occurs 7 to 10 days after the injection of heterologous serum, therefore several days after testing with antigen has been completed, but test subjects who have previously received the same heterologous antiserum may develop an accelerated form of local serum sickness before the experiment is complete. General serum sickness has not been observed but because of the possibility of its occurrence care is taken to limit the total amount of heterologous serum used in any experiment to about one milliliter or less.

The quantity of skin-sensitizing antibody is measured approximately by sensitizing sites with successive dilutions of serum in saline and taking

as the titer the highest dilution producing definite sensitization. Since various test subjects may give slightly different titers with the same antiserum, the results are not precise, and comparisons of the activity of different sera are accurate only if tested simultaneously in the same subject.

NATURE OF REACTIONS PRODUCED

The reactions produced in normal human skin by passive sensitization with guinea pig and rabbit antisera and subsequent tests with antigen are identical with the classical Prausnitz-Küstner reaction produced by passive sensitization with sera of allergic men, being characterized by an urticarial wheal with surrounding erythema appearing within 10 to 15 minutes and subsiding in an hour or two. The intensity of the reactions produced by sensitization with rabbit antisera has in many instances equaled those ordinarily produced with human allergic sera with wheals 2 cm. in diameter,[2] and activity has been demonstrable with dilutions of antiserum up to 1:1000, essentially as high as the usual titers of human sera. No delayed or hemorrhagic reactions of the Arthus type have been observed when amounts of rabbit anti-ovalbumin as high as 60 micrograms of antibody nitrogen have been injected intracutaneously and followed one or two days later with 50 micrograms of antigen into the same site.

SPECIFICITY OF EXPERIMENTAL SKIN-SENSITIZING ANTIBODIES

The specificity of this type of passive sensitization has been amply demonstrated by testing with related and unrelated antigens. For example, when sites were sensitized with antibody from a rabbit sensitized to horse serum, there was no reaction to tests with horse dander or guinea pig serum (Table 3). When these same sites were retested 24 hours later with horse serum, the antibody of the site previously tested with this antigen had been exhausted or neutralized, while the sites previously tested with other antigens remained active (17).

As with human skin-sensitizing antibodies, specific neutralization of

[2] Reactions with urticarial wheals over 2 cm. in diameter were recorded as + + + +, wheals 1.5 to 2 cm. + + +, 1 to 1.5 cm. + +, and those with distinct wheals less than 1 cm. as +.

TABLE 3

RABBIT ANTIHORSE SERUM 51, TESTED WITH OTHER ANTIGENS

Site	*Test-Subject*	*Tested with 0.1 mg. Protein N per ml.*	*Reaction*	*Reaction on Retest with Horse Serum 1:100, 24 Hours Later*
1	C	Low ragweed	0	++
2	C	Cottonseed	0	++
3	C	Horse dander	±	++
4	C	Horse serum[a]	++	±
5	C	Guinea pig serum[a]	0	++
6	C	Egg white	0	++
1	D	Horse serum[a]	++	
2	D	Horse dander	0	
3	D	Guinea pig serum[a]	0	

Source: Sherman, Stull, and Hampton, *J. Immunol.*, 1939, 36:455.
[a] Horse serum and guinea-pig serum 1:100.

antibody may also be demonstrated by mixing antiserum and antibody *in vitro* before injection into skin sites. When a rabbit anti-horse serum was mixed with varying concentrations of horse serum, the mixture with horse serum 1:100 produced passive sensitization equal to that produced by a mixture of antiserum and saline, while the site injected with the mixture containing undiluted horse serum was not sensitized (Table 4).

TABLE 4

NEUTRALIZATION OF SERUM 51

Site	*Mixture with Equal Parts of:*	*Protein Nitrogen mgm./ml.*	*Reaction to Test with Horse Serum 1:100*
1	Horse serum 1:100	0.1	++++
2	Horse serum 1:10	1.0	++
3	Horse serum concentrate	10.0	0
4	Saline		++++

Source: Sherman, Stull, and Hampton, *J. Immunol.*, 1939, 36:455.

THERMOLABILITY OF RABBIT SKIN-SENSITIZING ANTIBODY

Since thermolability to 56° C. has been considered one of the classical criteria of human skin-sensitizing antibody, the effect of heat on the skin sensitizing activity of human and of rabbit anti-horse sera was compared (21). The three samples of rabbit anti-horse serum and seven samples of serum from three cases of human serum sickness all showed roughly comparable activity in passively sensitizing human skin. After heating for four hours at 56° C., the skin-sensitizing activity of all the human sera was destroyed. That of the rabbit sera was not changed

TABLE 5

EFFECT OF HEATING FOUR HOURS AT 56° C. ON SKIN-SENSITIZING ACTIVITY OF HUMAN AND RABBIT ANTIHORSE SERA

	UNHEATED				HEATED			
Sera	*Conc.*	*1:10*	*1:100*	*1:1000*	*Conc.*	*1:10*	*1:100*	*1:1000*
Human								
B-1	++++	+++	++	0	0	0	0	0
B-2	+++	++	0		0	0	0	
M-1	++++	+++	++	0	0	0	0	
M-2	++++	+++	++	0	0	0	0	
M-3	+	+	0		0	0	0	
S-1	++	±	0		0	0	0	
S-2	++	±	0		0	0	0	
Rabbit								
51	+++	+++	++	0	+++	+++	++	0
16	++	+	+	0	++	+	+	0
4	++	=	+		+	+	±	0

Source: Sherman, Cooke, Crepea, and Downing, *J. Allergy*, 1948, 19:161.

(Table 5). In view of this difference of thermolability, the effects of heat upon the anaphylactic antibody as demonstrated by the passive Dale reaction and upon the precipitin titer were tested. The same human sera and six rabbit anti-horse sera all produced a positive Dale reaction when 2 ml. of unheated serum was injected into a guinea pig. After heating for four hours at 56° C. none of the human antisera sensitized guinea pigs while four of the six rabbit sera remained active (Table 6). The precipitin titers of the seven human sera were slightly higher than those of the hour rabbit anti-horse sera selected for comparison before heating. After heating, the precipitin activity of six of the human sera was destroyed and the 7th was reduced from 1:10,000 to 1:20, while those of

Table 6

Effect of Heating Four Hours at 56° C. on the Activity of Human and Rabbit Antihorse Sera in Sensitizing Guinea Pigs

Sera	*Dale Test with Unheated Serum*	*Dale Test with Serum Heated Four Hours at 56° C.*
Human		
B-1	+	0
B-2	+	0
M-1	+	0
M-2	+	0
M-3	+	0
S-1	+	0
S-2	+	0
Rabbit		
69	+	+
17	+	+
20	+	+
57	+	0
6	+	0
25	+	+

Source: Sherman, Cooke, Crepea, and Downing, *J. Allergy*, 1948, 19:162.

Table 7

Effect of Heating Four Hours at 56° C. on the Precipitin Titers of Human and Rabbit Antihorse Sera

Sera	*Titer of Unheated Serum*	*Titer of Serum Heated Four Hours at 56° C.*
Human		
B-1	1:5000	0
B-2	1:10,000	0
M-1	1:10,000	1:20
M-2	1:5000	0
M-3	1:2000	0
S-1	1:5000	0
S-2	1:5000	0
Rabbit		
69	1:3000	1:3000
17	1:3000	1:1000
20	1:1000	1:1000
57	1:2000	1:500

Source: Sherman, Cooke, Crepea, and Downing, *J. Allergy*, 1948, 19:162.

the rabbit sera showed little or no change (Table 7). It was therefore apparent that thermolability was a common property of a number of human antibodies rather than a specific characteristic of the human skin-sensitizing antibody. Although the rabbit antibody which passively sensitized human skin differed in thermolability from the human antibody producing the same reaction, this difference was apparently a species difference rather than a difference in the type of antibody.

DISTINCTION BETWEEN ANAPHYLACTIC AND SKIN-SENSITIZING ANTIBODY

In the studies already cited of sera of guinea pigs sensitized with alum-precipitated ragweed, it was apparent that anaphylactic antibody was often present without demonstrable skin-sensitizing activity. In these experiments, however, it was impossible to state whether the variations depended upon qualitative or quantitative differences in the antibody present. More conclusive evidence that anaphylactic and skin-sensitizing activity depended on different antibodies was furnished by the opportunity to study the skin-sensitizing properties of guinea pig anti-ovalbumin sera prepared in the Allergen Research Laboratory of the Department of Agriculture. In these antisera, Dr. E. J. Coulson had measured by an exacting technique of passive anaphylaxis, the amount of anaphylactic antibody expressed in the number of sensitizing doses (for a 300 gm. guinea pig) per milliliter of serum. Since all of the three sera which sensitized human skin were from guinea pigs which had survived challenging injections of antigen, a comparison was made of sera from this group (Table 8). The anaphylactic titers of the three sera sensitizing human skin were in the same range as those of the four sera showing no skin-sensitizing activity. In other words there was no correlation between the amount of anaphylactic antibody and that of skin-sensitizing antibody (18).

DISTINCTION BETWEEN PRECIPITIN AND SKIN-SENSITIZING ANTIBODY

A lack of correlation between precipitin titer (as determined by dilution of antigen or antibody) and skin-sensitizing activity was apparent in many of the studies with rabbit antisera (Table 2). For a more accurate evaluation of this relationship, quantitative precipitin studies were carried out on the sera of rabbits given multiple subcutaneous in-

TABLE 8

COMPARISON OF ANAPHYLACTIC AND SKIN-SENSITIZING ACTIVITY OF GUINEA PIG ANTI-OVALBUMIN SERA

Guinea Pig No.	*Anaphylactic Titer*[a]	*Skin-Sensitizing Titer*[b]
11.195	100	1:100
11.706	130	1:10
11.711	200	1:100
Average	143	Positive
11.160	40	0
11.180	133	0
11.181	100	0
11.707	400	0
Average	168	0

[a] Sensitizing doses per ml. [b] Highest dilution sensitizing human skin.

jections of crystalline egg albumin (20). As previously noted, 14 of these rabbits had received alum-precipitated antigen. The sera of all 14 sensitized human skin; the precipitin contents of the sera varied from 0.047 to 0.604 mg. of N per ml., with an average of 0.299. None of the sera from the rabbits given injections of dissolved egg albumin produced definite sensitization of human skin. The precipitin contents of five of these sera varied from 0.088 to 0.466 mg. of N per ml., with an average of 0.301. It was apparent that the precipitin content bore no relation to the skin-sensitizing antibody titer (Table 9).

As a result of the quantitative precipitin determinations, samples of antisera remained, from which 66–94 percent of the calculated precipitable antibody had been absorbed by a single addition of antigen. Eight of these partially absorbed sera, from rabbits which had shown definite skin-sensitizing activity, were compared with the corresponding untreated sera in serial dilutions in human skin. As shown in Table 10 the absorbed and unabsorbed sera from each rabbit showed essentially equal skin-sensitizing capacity, indicating that most of the precipitable antibody nitrogen could be removed without affecting the skin-sensitizing antibody. From two of the skin-sensitizing rabbit anti-ovalbumin sera, the precipitating antibody was completely absorbed by fractional additions of antigen. Two aliquots of each serum were used; from one a portion of the precipitin was absorbed by a small addition of antigen, then the two samples were simultaneously carried through five or six

TABLE 9

LACK OF CORRELATION OF PRECIPITIN CONTENT AND SKIN-SENSITIZING ACTIVITY OF RABBIT ANTI-OVALBUMIN SERA

SERA OF RABBITS IMMUNIZED WITH ALUM-PRECIPITATED ANTIGEN

Serum No.	*Precipitin N* (mg. per ml.)	*Value of R Calculated*	*Reactivity on Passive Transfer to Human Skin Diluted:* *Undiluted*	*1:10*	*1:100*
9	0.258	7.5	+++	++	±
10	0.381	6.1	++	+	−
11	0.358	9.0	+	+	−
12	0.595	9.3	+	+	−
13	0.531	10.2	+	+	−
14	0.604	10.0	++	+	−
15	0.338	9.0	+++	+++	+++
16	0.553	8.9	+	−	−
130	0.170	8.4		++	+±
132	0.047	7.5		+++	++
135	0.071	10.0		++	++
164	0.102	10.6	+±	±	±
165	0.087	7.5	++	+	+
191	0.083	9.1	+		

SERA OF RABBITS IMMUNIZED WITH DISSOLVED ANTIGEN

Serum No.	*Precipitin N* (mg. per ml.)	*Value of R Calculated*	*Reactivity on Passive Transfer to Human Skin Diluted:* *Undiluted*	*1:10*	*1:100*
1	0.088	6.7	−		
3	0.420	7.4	−		
4	0.446	8.8	−		
5	0.193	6.2	−		
8	0.359	8.4	±		

Source: Sherman, Menzel, and Seebohm, *J. Exp. Med.*, 1950, 92:194.

subsequent additions of antigen until the aliquot which had been subjected to one more antigen addition showed no precipitate, while the second showed a slight precipitate. Thus one portion contained a trace of precipitin, the other no precipitin but presumably a trace of antigen. The skin-sensitizing activity of both portions were compared with that of the original serum in corresponding dilutions on normal human skin.

TABLE 10

COMPARISON OF REACTIVITY OF UNABSORBED AND ABSORBED SERA ON PASSIVE TRANSFER TO HUMAN SKIN

Serum No.	*Precipitin N Removed (Percent)*	*Reactivity on Passive Transfer to Human Skin Diluted:*			
		1:200	1:400	1:800	
9	0	+	±	−	
	89	+	±	−	
		1:2	1:10	1:20	1:40
11	0	+	+	±	−
	94 ± 4	+	+	±	−
		1:2	1:4	1:8	1:16
14	0	+	+	±	−
	66	+	+	±	−
		1:80	1:160	1:320	
15	0	+	±	−	
	92 ± 6	+	±	−	
		1:10	1:100	1:200	
130	0	+++	±	−	
	68	+++	±	−	
		1:100	1:1000	1:2000	
132	0	++	±	−	
	82 ± 5	++	±	−	
		1:10	1:100	1:1000	
135	0	++	±	−	
	75	++	+	±	
		1:20	1:40	1:80	
164	0	+	±	−	
	79	+	±	−	

Source: Sherman, Menzel, and Seebohm, *J. Exp. Med.*, 1950, 92:195.

In both instances, the two samples of adsorbed sera and the original serum passively sensitized human skin in the same dilutions (Table 11). It was apparent that the skin-sensitizing antibody of these sera remained unaffected when the precipitating antibody had been completely removed by fractional adsorption.

From Serum 14, which showed moderate skin-sensitizing activity, all the precipitable antibody was removed by a single addition of the calculated amount of antigen. The supernatant, which showed no detectible excess of antigen or antibody, failed to sensitize the skin of either of two subjects tested. The skin-sensitizing antibody apparently was removed along with the precipitin, if this was accomplished by a single addition of the calculated equivalent amount of antigen. Thus the skin-sensitizing antibody showed the properties of an incomplete or so-called "univalent" antibody, while the true precipitin appeared to take no part in the passive sensitization of human skin.

TABLE 11

COMPARISON OF THE DILUTION TITERS OF UNABSORBED AND STEP-WISE COMPLETELY ABSORBED TRANSFERRING SERA

	REACTION ON TEST WITH CRYSTALLINE OVALBUMIN OF SKIN SITES SENSITIZED WITH 0:1 ML. OF:					
	Serum 9			*Serum 14*		
Dilution 1:	*Unabsorbed 9*	*Absorbed 9A*	*Absorbed 9B*	*Unabsorbed 14*	*Absorbed 14A*	*Absorbed 14B*
1:5.5				+	+	+
1:8	+++	+++	+++	+	+	+
1:16	+++	+++	+++	±	±	±
1:32	+++	+++	++	0	0	0
1:64	++	++	++			
1:128	±	+	+			
1:256	±	±	±			
1:512	0	0	0			

Source: Sherman, Menzel, and Seebohm, *J. Exp. Med.*, 1950, 92: 196.

This is of interest in comparison with the findings of Kabat and Benacerraf (22, 23) in studies of passive sensitization of rabbits and guinea pigs with rabbit anti-ovalbumin sera. They found that the precipitating and "univalent" antibodies were equally active in producing passive anaphylaxis in the guinea pig but that the precipitin showed much greater activity than non-precipitating antibody in producing the Arthus reaction in guinea pigs and rabbits. Thus the type of skin sensitization produced in human skin by these rabbit antisera differs from the typical Arthus reaction in the immune mechanism as well as in its pathologic features.

The separation of precipitin and skin-sensitizing antibody in rabbit

antisera helps to establish the qualitative difference between these two types of antibody and, indirectly, to refute the frequently expressed view that failure to demonstrate precipitin in skin-sensitizing sera from human allergic patients is due simply to the low concentration of antibody present.

No evidence has been obtained as to whether or not all incomplete or "univalent" antibody is skin-sensitizing antibody. No attempt has yet been made to correlate the amount of "univalent" antibody in a sample of serum with its skin-sensitizing potency, nor have rabbit anti-ovalbumin sera which did not sensitize skin been studied for the presence of "univalent" antibody. Since passive sensitization of skin presumably depends upon the presence of antibody with a special affinity for this tissue, it appears probable that the skin-sensitizing antibody may be only a portion of a variety of antibodies whose reactions *in vitro* fit the criteria of "univalent" antibody.

Since the ratio of antigen to antibody N precipitated at the point of equivalence (R in Heidelberger's equation (24)) is a measure of the combining capacity of the antibody in a serum, it was of interest to compare the calculated values of R in rabbit anti-ovalbumin sera which did and did not sensitize human skin. The values of R in 14 sera showing skin-sensitizing activity varied from 6.1 to 10.6, with the average 8.8, while those of five precipitating sera which did not sensitize human skin ranged from 6.2 to 8.8, averaging 7.5 (Table 9). Since the values in both groups fell in the same general range, and since the individual sera showing the highest (10.6) and lowest (6.1) R values had essentially equal skin-sensitizing activity, there was no apparent relationship between the R value and the presence of skin-sensitizing antibody.

SUMMARY

Antibodies producing passive sensitization of human skin have been produced in rabbits and guinea pigs by stimulation with such antigens as egg albumin, horse serum, bovine albumin, and extracts of ragweed and grass pollens. By most techniques of sensitization, only a fraction of animals developing precipitins show skin-sensitizing activity. Optimal sensitization procedures have not been determined, but it is apparent that alum-precipitated antigens are more effective than soluble antigens.

These skin-sensitizing antibodies are demonstrable by the usual

Prausnitz-Küstner technique and the reactions obtained are identical with those produced by human allergic sera.

Most of the rabbit and guinea pig antisera showing skin-sensitizing activity also contain anaphylactic antibodies and precipitins, but the degree of skin-sensitizing activity is not related to precipitin content or activity in passive anaphylaxis. By fractional absorption with antigen, all precipitating antibody may be removed from such sera without affecting their skin-sensitizing activity.

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Chapter 9

SKIN-SENSITIZING ANTIBODY AND ALLERGY AND RESISTANCE TO INSULIN

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The serum of a large proportion of persons having hay fever and asthma has the capacity, when injected into the skin of suitable recipients, to confer a localized change, so that the subsequent injection into the site of a substance capable of inducing whealing and erythema in the skin of the donor will likewise cause this reaction in the skin of the recipient. The terms skin-sensitizing antibody, allergic antibody, and atopic reagin have been applied to the factor or factors in the serum responsible for this reaction.

As the ability of skin-sensitizing antibody to confer sensitivity of the so-called "immediate type" on non-sensitive skin is not peculiar to this antibody, is one justified in assigning this antibody to a special category? Is it not simply an antibody like any other, perhaps differing as antibodies in general are known to do in its capacity to produce visible reactions in the test tube, to sensitize animals passively, to fix complement, and so on? This view has been well expressed on many occasions, and it is in harmony with what is often referred to as the Unitarian Theory of antibody (1, 2). The opposing view is widely held, however, that skin-sensitizing antibody is sufficiently different from the antibodies with which immunologists have been chiefly concerned to warrant the special designation "atopic," as suggested by Coca (3). It is true that today there is increasing evidence that many of the criteria which had been earlier considered as peculiar to skin-sensitizing antibody require considerable modification, and the differences which appeared so sharp when first described are now becoming more and more difficult to define (2, 4, 5). Nevertheless, there is a combination of properties which seem to characterize this antibody and set it apart from others that are known.

Skin-sensitizing antibody fails to produce *in vitro* the reactions which are commonly applied to the detection of other antibodies, namely precipitation or fixation of complement. Most attempts to sensitize ani-

mals have failed, with the exception of monkeys (6). The Schultz-Dale reaction has likewise been unsuccessful. It has been difficult to induce the formation of skin-sensitizing antibody artificially in man (7). Occasionally, individuals receiving treatment with therapeutic agents such as insulin, liver extract, and certain drugs develop skin-sensitizing antibody associated with a high degree of allergy. Skin-sensitizing activity is also often found in the blood of those who have recently received animal serum for prophylactic or therapeutic reasons. The sensitization conferred on a skin site by skin-sensitizing antibody persists for as long as a month and possibly longer (8). Although this may be a unique property of skin-sensitizing antibody, there is a possibility that the capacity of antibody produced in animals to confer a persisting sensitization on skin may have been overlooked. Its demonstration would require the use of homologous sera, as the production of antibody for heterologous serum would presumably abolish an established sensitization. The prompt inflammatory reaction which takes place when the appropriate antigen is injected into the skin of those possessing skin-sensitizing antibody is short-lived, and is rarely, if ever, followed by gross evidence of tissue damage. On the other hand, the injection of antigen into the skin of animals with high degrees of sensitivity associated with precipitins is followed by the Arthus reaction, an acute inflammatory lesion with thrombosis of blood vessels (9). This is not only readily produced in animals, but has been occasionally seen in man following the second of two suitably spaced injections of horse serum. In view of the high degrees of allergy frequently encountered among those with circulating skin-sensitizing antibody, the frequency with which such individuals receive injections of antigen, and the intensity of the local reactions, it is surprising that this reaction is so rare.

As the behavior of any antigen-antibody system is dependent not only on the nature of the antibody, but also on the attributes of the antigen, the antigen alone might be held responsible for the peculiarities of skin-sensitizing antibody. The role of an antigen as related to the skin-sensitizing activity of its antibody is illustrated by the experience with rabbit antiserum for egg albumin on the one hand, and antipneumococcus serum on the other, both having the usual characteristics of conventional antibody. Attempts to sensitize human skin with rabbit anti-ovalbumin serum have given irregular results (8, 10, 11). On the other hand a hitherto important clinical test (12, 13) depended on the ability

of rabbit antipneumococcal serum, when injected intravenously to induce the "immediate" type of sensitivity to the type-specific polysaccharide of the pneumococcus. Thus these two antibodies having the same source, and being similar in other respects, differ in their tendency to induce skin sensitivity. Presumably this difference is in some way connected with the nature of two antigens. High degrees of allergy associated with skin sensitizing antibody is often found in persons with worm infestation (14), again suggesting the importance of the antigen in determining the type of immunologic response.

There is ample evidence to indicate that animals develop antibody readily for antigens in ragweed pollen, probably the commonest and most widely studied of the antigens giving rise to hay fever and asthma. However, the antibody developed by sheep (15) and rabbits (16) for ragweed behaves as a precipitin and, when tested in human skin, as a "blocking" antibody. A similar result is obtained when ragweed extract is injected into non-allergic individuals (7), although precipitins have not been observed. Normal persons injected in this manner do not become allergic. Certainly the nature of the allergen is not the sole determinant for the production of skin-sensitizing antibody.

When precipitins are present in human serum the situation is clearly unlike that found in hay fever and asthma, where very active serums give no precipitate. There is a danger in assuming that a serum which can induce skin sensitivity necessarily contains the skin-sensitizing antibody. An example of this is the induction of skin sensitivity in man with ascaris antigen (17). The precipitins associated with skin-sensitizing activity in the serum of patients with serum disease present a similar problem. In instances of this kind it is difficult to show that precipitins are not responsible for the skin reactions observed.

Most antibodies induced artificially in man or animals tend to decrease and disappear in the course of a few months after exposure to the antigen is stopped. On the other hand, the skin-sensitizing antibody in man often persists in undiminished amounts for many months without known exposure to the antigen. The word "known" is the Achilles' heel of the previous sentence, because it would appear impossible to prove that exposure is lacking, as for example, in the intervening eight or nine months between pollen seasons. Exposure to pollen during the pollen season brings about an increase in the titer of skin-sensitizing antibody (18) as does also a series of therapeutic injections of allergenic extract

(18, 19, 20). However, the latter induces a marked increase in the neutralizing capacity of the serum as well, a change which either does not occur in the ordinary exposure or which occurs to a very small degree. The possibility that exposure may induce the formation of the "blocking" or heat stabile antibody should not be lost sight of, however, in experiments designed to test the neutralizing capacity of skin-sensitizing antibody. The increase in titer of skin-sensitizing antibody during a course of injections with the allergen is not surprising in view of the rather strong evidence indicating that the production of skin-sensitizing activity depends on exposure. The maintenance of a relatively stable level of antibody is, however, difficult to explain in view of the tendency for the titer of most antibodies to fall in the absence of antigenic stimulation and to rise upon exposure.

The known distribution of skin-sensitizing activity in the population strongly indicates that the ability of individuals to develop this antibody varies widely. In addition to its occurrence in a relatively high proportion of subjects with allergic disease, a high degree of allergy associated with skin-sensitizing activity occasionally crops up among the vast number of patients receiving one or another biologic preparation or drug. Although there seems to be a constitutional or hereditary predisposition to develop skin-sensitizing activity among those having hay fever and asthma, among those relatively few patients who develop high degrees of allergy while under treatment with insulin or liver extract, hay fever and asthma are not common (21). The development of allergy under these circumstances is entirely unpredictable. One is reminded of the surprisingly low incidence of erythroblastosis fetalis, some twentyfold less frequent than would be expected on the basis of what is now known (22). In view of the wide success in inducing antibody formation (23) and allergic reactions (24, 25) with adjuvants composed of oil emulsions and acid-fast organisms, one wonders whether the development of antibody of whatever kind, occurring as it does in an irregular and unpredictable manner, may not depend, in part at least, on purely accidental events. For example, the development of a high degree of allergy to the sulfonamide drugs was surprisingly frequent among patients with eczematous skin lesions receiving topical treatment with these drugs (26, 27, 28). It is possible that incidental infections, localized damage, or in the case of injected therapeutic agents, localized infection or

trauma from preceding injections may bring about every so often just those changes in tissue which predispose to antibody formation.

Perhaps the most difficult aspect of the antibody responsible for the skin-sensitizing activity of certain human sera is whether or not this antibody is capable of interfering with some property of the antigen by combining with it. Attempts to determine whether, in mixtures of antigens and skin-sensitizing antibody, these two components migrate together or separately in an electric or strong gravitational field led to the conclusion, based on rather indirect evidence, that they did not migrate together, and that no combination of antibody and antigen had occurred under the conditions of the experiment (29).

Attempts to detect neutralization of antigen by skin-sensitizing antibody have for the most part been unsuccessful. However, Schmidt and Lippard (30) claim to have demonstrated such an effect. Their work was done when the probable existence of two separate antibodies in treated allergic individuals was less widely recognized than it is today, and the possibility cannot be excluded that recent exposure to the antigen in those subjects from whom the sera were obtained had occurred, and that the neutralization observed was of the kind seen in the serum of patients treated with injections of allergenic extracts. Walzer (31) has made similar observations, and Loveless (32) has obtained rather irregular evidence of such neutralization by titrations made in the conjunctival sac. In order to be certain that the skin-sensitizing antibody is actually responsible for the observed neutralization of antigen, it is essential to show that the skin-sensitizing antibody is the only antibody present in the serum under test. This is difficult to do with antibody for the pollen antigens.

The question as to whether or not skin-sensitizing antibody neutralizes the antigen might be settled, if some unique property of the antigen were interfered with specifically by the antibody in a manner analogous to the neutralization of diphtheric toxin by diphtheric antitoxin. Kuhns and Pappenheimer are studying this problem in individuals with induced immediate-type skin reactions to diphtheria toxoid (33).

In patients with allergy to insulin the capacity of skin-sensitizing antibody to neutralize the antigen can be advantageously studied, and a considerable amount of work has been done. Clinical experience has clearly shown that, although patients whose sera possess skin-sensitizing

activity for insulin may be resistant to the physiologic action of insulin, this is not invariably the case; in fact, one has the impression that in patients allergic to insulin, resistance is more often absent than present.

A patient recently studied by us (34) developed generalized urticarial reactions following the injection of insulin to control mild diabetes. The intravenous injection of 0.1 unit of commercial crystalline insulin per kilogram, the usual dose for an intravenous tolerance test, caused the expected fall in blood sugar and also generalized urticaria. The patient's serum had skin-sensitizing activity for insulin. Therefore, this patient was allergic, but not resistant. Were the commercial product known to be pure insulin, an experiment such as this would appear to settle the question as far as insulin is concerned. However, Jorpes (35, 36) has administered recrystallized insulin to patients who were allergic to the commercial preparation and found that insulin recrystallized several times produced no allergic reaction. He concludes that insulin itself is not the substance to which such patients are allergic. Many details are omitted in this study so that one cannot always precisely know what type of reactions occurred in each instance, nor just how they were related to the administration of the various insulins used. Local swelling is often seen in the first weeks or months of treatment with insulin (37). Such reactions tend to disappear with the passage of time. Attempts to demonstrate skin-sensitizing activity for insulin in such patients have in general been few, nor did Jorpes do any studies along these lines. The question may be raised whether the type of allergy seen in the great majority of such patients is the same as that in which skin-sensitizing activity is demonstrable in the serum. Clinically, most of these reactions appear to be entirely different. Local swelling follows the injection of insulin after several hours and reaches its peak in about twenty-four hours. Local reactions associated with the presence of skin-sensitizing activity, on the other hand, characteristically appear within a few minutes, are usually subsiding or have disappeared within a few hours, and are often accompanied by systemic allergic manifestations.

The crystalline insulin available commercially in this country appears to possess in addition to its hypoglycemic activity, a hyperglycemic factor demonstrable only by intravenous injection into man or animals (38, 39). The hyperglycemic factor differs from insulin in that it is derived from the alpha cells in the islets of Langerhans and is not inactivated by cysteine or alkali. Its presence in the pancreas of dogs rendered diabetic

with alloxan probably accounts for the greater insulin requirement before, as compared with after, pancreatectomy in such animals. If commercial insulin contains a hyperglycemic factor distinct from insulin itself, as well as allergenic factors removable by recrystallization as described by Jorpes, then, before a conclusion can be reached regarding neutralization of insulin by skin-sensitizing antibody, it is essential to show that the skin-sensitizing activity seen in patients who are allergic to insulin is directed against the hypoglycemic factor.

In earlier reports (40, 41, 42) a patient was described who was both allergic and resistant to insulin. Evidence for the existence of two antibodies in this patient was presented, a skin-sensitizing antibody and an insulin-neutralizing antibody. Studies indicated that the skin-sensitizing antibody did not interfere with the physiologic action of insulin, or did so to such a small degree that interference was not detected. This patient was given courses of insulin at widely spaced intervals. Relatively small doses of insulin, which at first caused generalized urticaria, were given at the beginning of each course and in a few days ordinary therapeutic doses could be tolerated. At this time such doses of insulin caused a fall in blood sugar. Resistance appeared about fourteen days after a course of commercial crystallin insulin was begun, and this was associated with the appearance of an insulin-neutralizing factor in the serum, presumably an antibody. In this period the patient had changed from being exquisitely allergic to insulin, suffering generalized urticaria following the endermal or subcutaneous injection of small amounts of insulin, to being capable of tolerating the intravenous injection of thirty units or more without an allergic reaction. Titration by the dilution method indicated that the amount of skin-sensitizing antibody was the same before the course of insulin was begun and after resistance had developed. However, the serum now had the capacity, which it formerly lacked, to interfere with the hypoglycemic effect of insulin in mice. Therefore, a "blocking" antibody appearing in response to injected insulin and demonstrably anti-insulin in its activity seemed to protect the patient from what otherwise would have been the dire consequences of intravenously injected insulin. The parallelism between this and the situation in treated hay fever, wherein skin-sensitizing and an induced "blocking" antibody coexist, is obvious. Sherman (43) has made similar observations in another patient with allergy and resistance to insulin. In the patient under consideration, the antibody which interfered with

the hypoglycemic action of insulin would logically also interfere with the reaction in the tissues between insulin and skin-sensitizing antibody. It is difficult to escape the conclusion that the skin-sensitizing activity specifically sensitized the skin for physiologically active insulin and not an associated contaminant. The possibility cannot be ruled out, however, that something in commercial insulin other than the hypoglycemic factor accounted for the allergic reactions and that "blocking" antibody for this was produced at the same time as the insulin-neutralizing antibody.

Other observations may have a bearing on this point. The patient just described reacted by skin test to human insulin, and skin-sensitizing activity for this insulin was present in the serum. However, the situation with regard to human insulin differed from that regarding commercial insulin in two respects: first, an acute generalized allergic reaction followed the intravenous injection of this insulin, the first injection of the kind that the patient had received, and the reaction occurred at a time when, as described above, the patient had become resistant and did not react with allergic manifestations when commercial insulin was injected intravenously; second, human insulin had a pronounced hypoglycemic action. Thus it is tempting to conclude that the lack of neutralizing antibody for the hypoglycemic factor in the preparation of human insulin left the patient unprotected against its allergic effects. This again suggests that the skin-sensitizing antibody was directed against the hypoglycemic factor, but was incapable of interfering with its action.

If one assumes that the skin-sensitizing antibody in the serum of this patient was not directed against the hypoglycemic factor, then one must postulate an allergenic contaminant of a different nature in the preparation of human insulin from that presumed to be present in the commercial preparation, and the patient must have had skin-sensitizing antibody for each of these. On the other hand, the occurrence of an allergic reaction upon the injection of human insulin for the first time is more easily explained by assuming that both the human and commercial insulins had immunologic properties in common. This also suggests that the skin-sensitizing antibody had a lower degree of specificity than the neutralizing antibody. It is possible that the skin-sensitizing antibody and the insulin-neutralizing antibody combined with different groupings on the insulin molecule. Indeed, this might also explain the difference in the behavior of the two types of antibody with respect to the hypoglycemic activity of insulin.

SUMMARY

There is still much to be done before the skin-sensitizing antibody as seen in man is placed on the firm footing enjoyed by antibodies which form precipitates, fix complement, or neutralize toxins. The methods available for the study of this antibody, dependent as they are on tissue reactions, are rather unsatisfactory from a quantitative point of view.

A number of characteristics of skin-sensitizing antibody as seen in man set it apart from other known antibodies and would appear to warrant, for the time being at least, a special designation for this antibody. Its frequent occurrence in individuals with hay fever and asthma contrasts markedly with its absence in a large proportion of the population. Satisfactory evidence that this antibody consistently combines with the antigen so as to alter some property of the latter or to interfere with its capacity to produce an allergic reaction is lacking. On the contrary, the experience with allergy to insulin indicates that this antibody may not interfere with the hypoglycemic action of the hormone.

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Chapter 10

IMMUNOLOGICAL REACTIONS MEDIATED THROUGH CELLS

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In a consideration of immunological reactions that are mediated through cells, two lines of thought are obviously entwined, namely the role of host cells in the production of one or more varieties of antibody, and the behavior of cells—*in vitro* or *in vivo*—when tested by the administration of antigen. Put otherwise, our task might be conceived as being a review of instances in which cells of the individual who has been exposed to antigenic stimulus seem to be uniquely involved in and responsible for the occurrence of antigen-antibody reactions.

Divers studies have all pointed to a participation of cells in reactions that, because of manifest specificity, have appeared to be immunologic in nature. The occurrence of localized areas of reaction, the so-called "fixed" eruptions, seen in allergic man has pointed to a cellular role in production of the lesions. Again, the early and neglected studies of Bail on the transfer of tuberculin sensitivity suggested the importance of host cells. In another area, the study of cells from the tuberculous host in tissue culture, initiated by Rich and Lewis in 1932 (1) and now well confirmed, showed that the presence of tuberculin had a profound effect upon the macrophages, causing them to cease peripheral migration and to undergo death. My own concern dates from the winter of 1940, when in studies with the late Karl Landsteiner on the sensitization of animals with simple chemical compounds it was found that the production of a specific dermal sensitivity was a function of washed exudate cells donated by sensitized guinea pigs to non-sensitized individuals of this species (2, 3). Still another area of investigation opened as a consequence of the studies of McMaster, and Ehrich and Harris, and White and Dougherty, implicating cells of the lymphoid series in the production of antibody. Finally, we may mention the direct and speedy lytic effect of tuberculin on a proportion of the lymphocytes of tuberculous indi-

viduals shown so beautifully by Favour and termed "lympholysis" (4, cf. 34).

From all of these studies, a pattern is slowly beginning to appear. For reason of brevity, it will be necessary to abandon or scatter much related work, and for bibliography and orientation to refer again to the paper presented by Dr. McMaster (Chapter 2, above).

I should like first to present evidence that washed cells donated by sensitized guinea pigs confer dermal sensitivity upon normal animals. The cells can be obtained from exudates induced in the peritoneal cavity, the leukocytic cream or "buffy coats" of peripheral blood, the spleens, and the lymph nodes (5); the effectiveness of the collected, washed cells from the first three sources has shown a parallelism with the content of lymphocytes. Upon intraperitoneal injection in amounts of 0.25 to 1.0 ml. of moist, packed cells, the dermal sensitivity becomes manifest within two to three days, but within about 16 hours if the intravenous route has been employed. Since the reactivity sought is of the "delayed" type, the test applications of allergenic excitant or of antigen are made a day in advance of intended readings. It may be added that the necessary volume of cells is inversely proportional to the degree of hypersensitiveness attained by the donors.

Case 1. The establishment of dermal sensitivity by means of washed white cells.[1] A normal guinea pig received by the intravenous route washed cells that had been recovered from the peritoneal cavities of seven guinea pigs sensitized to *o*-chlorobenzoyl chloride; paraffin oil had been injected 48 hours before, in order to induce the necessary cellular accumulation. The recipient was first tested nine hours later by placing on the skin one drop of olive oil containing 15 percent *o*-chlorobenzoyl chloride. A well-developed positive contact reaction was observed on subsequent inspection (16 hours later). The same result followed two further contact tests, made at 29 and 53 hours after injection of the cells. The animal did not react to a non-related chemical allergen, namely picryl chloride.

Case 2. The establishment of dermal sensitivity by means of cells secured from lymph nodes. As in Case 5b (*q.v.*), 1.0 ml. cells were recovered, washed and injected, 0.36 ml. intravenously, the remainder intraperitoneally. Tested on the third following day by application to the skin of one drop of olive oil containing 1 percent picryl chloride, the recipient animal showed an intense reaction 21 hours later.

[1] The descriptions here presented are given in lieu of the lantern slides that were projected at the session.

Case 3. Tuberculin-sensitivity transmitted by cells, but not by heated cells (5). Cells were obtained as in Case 1 from ten guinea pigs that had been sensitized by injection, intramuscularly, of heat-killed tubercle bacilli suspended in hydrocarbon. The pooled washed cells were divided into two equal portions; one portion was injected into recipient No. 1 by the intravenous route; the other portion was heated at 48° C. for 15 minutes and injected similarly into recipient No. 2. These two animals were given tuberculin skin-tests with Old Tuberculin and the tuberculoprotein preparation designated "PPD–67–2," both at four hours and at 26 hours after injection of the cells. Recipient No. 1 gave positive skin-tests to all 4 injections, recipient No. 2 failed to react.

Case 4. Local changes induced at the site of deposition, into the skin, of cells taken from tuberculin-sensitive guinea pigs: the experiment of Metaxas and Metaxas-Buehler (6). While the original experiment was made with cells of tuberculous guinea pigs, the one shown here was done with cells from guinea pigs sensitized to heat-killed tubercle bacilli suspended in hydrocarbon. Two recipient animals were each injected intracutaneously with 0.05 ml. of an approximately 50 percent suspension of pooled cells from peritoneal exudates of (*a*) sensitized guinea pigs and (*b*) normal guinea pigs. After 48 hours, 0.25 ml. of Old Tuberculin was injected subcutaneously and a few hours later 1.25 ml. Old Tuberculin was injected into the peritoneal cavity. In one of the recipients, namely that one of the pair which happened to exhibit a definite systemic reaction, necrosis analogous to that seen at the center of strong tuberculin reactions developed gradually, over a period of three days, within the area containing the cells. While this necrosis was pronounced at the site of the "sensitive" cells, definite involvement to a minor degree occurred as well at the site of the normal cells. Alterations in the injected sites of the second recipient were minor. Elements peculiar to the Shwartzman phenomenon may be involved, for recipients that had been injected with normal cells alone did not exhibit such a change following the injection of Old Tuberculin. It should be reiterated that the experiment of Metaxas was not followed precisely.

Thus we have seen several characteristics of the cellular transfer of hypersensitiveness. The effect is independent of any peculiarity of method used in sensitization of the donors; it is specific; it is valid both for drug sensitivities, whether tested by contact or injection, and for the delayed type of tuberculin allergy; the process does not take place if the cells are damaged by freezing or mild heating. Further, while it has not been possible to prepare active extracts from the cells, the experiment of Metaxas (6) shows that the cells themselves can give local evidence of their contribution.

It may be recalled that the above experiments have been confirmed, by Haxthausen (using cells of the thymus) for drug sensitivity (7), and for tuberculin hypersensitivity by Cummings, Hoyt, and Gottshall (8) and Kirchheimer and Weiser (9) with the use of cells from peritoneal exudates induced by paraffin oil, and by Stavitsky (10) with cells of exudates, of spleens and of lymph nodes. In these several cases the transfer was made from guinea pig to guinea pig.[2] In addition, Lawrence (12) finds that non-sensitive human subjects may similarly be made sensitive to tuberculin by the transfer of cells.

One further and important step was taken by Kirchheimer, Weiser, and Van Liew (13), who reported that the transfer of cells in the guinea pig was accompanied by a systemic reactivity to tuberculin as well as dermal sensitivity.

Repeatedly, attempts have been made to detect circulating antibody in the serum of recipient guinea pigs following the transfer of washed cells. Many such experiments were unfruitful; it even was found that the introduction, into the blood stream, of artificially conjugated antigens, prepared with soluble proteins and the corresponding simple chemical substances, not only failed to produce evidence of anaphylaxis but did not cause evident diminution of the existing level of dermal sensitivity. Finally, however, the use of splenic cells in increased volume led to the finding of a positive Schultz-Dale reaction in the uterus of the recipient animal, and the way was opened for a new series of studies. Not only splenic cells but likewise cells teased from the lymph nodes were found effective.[3]

Case 5. Acquisition of the anaphylactic state following the transfer of washed cells. (*a*) *Cells of splenic origin.* From a larger pool of cells obtained by mincing the spleens of guinea pigs rendered highly sensitive to picryl chloride in a special manner (15), 0.8 ml. of well-washed cells was injected into a normal female guinea pig (270 gm. body weight), half being given intravenously, the remainder intraperitoneally. Forty hours later, the uterine horns were removed at operation, and were found to be anaphylactically sensitive to picrylated casein as test antigen.

(*b*) *Cells from lymph nodes.* Sensitization and mode of experiment as in (*a*): lymph nodes were teased apart under fluid, the cells streaming from

[2] The role of cells in the transfer of tuberculin sensitivity in the rabbit (11) has not been clearly elucidated. [Since the above was written, Wesslén (36) has reported successful experiments with lymphocytes of the rabbit thoracic duct.]

[3] Cf. (14), which appeared subsequent to the Symposium.

the tissue being passed through a fine-meshed sieve and washed; 0.6 ml. cells were injected, 0.24 ml. intravenously, the remainder intraperitoneally. The uterine horns of the recipient female guinea pig were shown on the 6th following day to be anaphylactically sensitive as tested with picrylated casein according to the Schultz-Dale technique.

Case 6. Failure of splenic cells that have been damaged by freezing to induce the anaphylactic state. A large amount of cells (3.4 ml.) was obtained from the minced spleens of guinea pigs sensitized as in Case 5a. The pool of washed cells was suspended and divided into five equal portions. Three portions in stoppered Pyrex test tubes were subjected to a cycle of three freezings (in alcohol-dry ice) and thawings. The entire content of each tube was then resuspended and injected into the peritoneal cavity of a normal female guinea pig. The various recipients were sacrificed between 11 and 15 days later. The paired uterine horns were mounted simultaneously in one chamber of a Schultz-Dale apparatus, and the sensitivity to contact with picrylated casein was tested. The tissues of each of the animals receiving washed but viable cells responded at once with identical contractions, but the uterine strips from the three animals receiving frozen cells failed to react. The time before testing the recipients was prolonged in order to foster active sensitization in the event that antigen had been transmitted along with the cells.

Case 7. Failure of extracts of cells to replace cells in development of antibody. From one pool of splenic cells, two recipient guinea pigs received 0.7 ml. cells, partly by the intravenous, partly by the intraperitoneal route. Antibody was detectable in the circulation of one animal at 48 hours, in the other at subsequent examination on the fourth day, and both showed systemic anaphylaxis when tested on the 10th day. In contrast, the extract of 1.85 ml. cells, of the same pool, made at 4° C. and injected into a third recipient, failed to lead to the appearance of antibody by the seventh day, and this animal remained free of symptoms when tested for systemic anaphylaxis one day later. The entire yield of cells from the lymph nodes of these very same donors, 2.0 ml., was extracted similarly, the fluid being injected into a fourth female guinea pig as recipient: neither tissue nor systemic anaphylaxis had developed by the 8th day, and there was no antibody in the serum. In still another experiment, 2.4 ml. of lymph node cells were collected, the extract of 1.35 ml. having no detectable effect on recipient No. 5 within 10 days, whereas 1.0 ml. of viable cells injected into recipient No. 6 led to the appearance of antibody by the third day, and to definite tissue and systemic anaphylaxis.

From the foregoing, a parallelism is evident with the conditions attending the transfer of dermal sensitiveness, with respect to similarity

of the sources of effective cells, the failure of the process when the cells are damaged, and also inability to obtain parallel results by means of extracts of the cells.

The positive results obtained by the Schultz-Dale technique on uterine horns of the cell-recipients now had to be related to the presence of circulating antibody, so that the onset and variations in concentration of antibody could be watched in single recipients. Fortunately a basic method for studying circulating antibody had been developed previously, employing reactions of the Prausnitz-Küstner type in guinea pig skin (16). With some modifications, namely injecting the test antigen intravenously and establishing the proper concentration of antigen to be administered, it became possible to determine antibody concentrations of approximately the order of 0.0003 mg. antibody N/ml. In this way, antibody could be measured in a comparative sense, and results corresponding to but independent of Dale tests became obtainable.

A series of appropriately spaced ear bleedings was made on individual recipients, each sample being approximately 0.8–1.0 ml. of blood, and the separated serum sufficed for the assays. The various samples were injected into the skin of normal guinea pigs in 0.1–0.15 ml. amounts, and 18 hours later the antigen (picrylated casein) was injected intravenously in appropriate quantity: readings were made between three and 15 minutes later, at the time of maximal reaction.

The time at which antibody appears in the circulation varies with the amount of cells, the route by which they are introduced, and other factors (see table). Antibody has been detected indubitably between 27 and 40 hours, but more often (because of the mode of sensitization of the donors) at the third day. Irrespective of the time of first appearance, in all instances the concentration is ascending up to the fifth or sixth day; it then becomes stabilized for some days, and thereafter slowly recedes. In the early appearance of antibody, and in negation of the process by freezing and thawing of the cells, there is strong suggestion that the effect does not reflect an active sensitization owing to transfer of the original antigen along with the host cells; this possibility could further be excluded entirely as an explanation of the observed results, for in the case of sensitizations effected with picrylated erythrocyte stromata of the guinea pig, the antigen is extremely inefficient and does not by itself lead to the formation of detectable antibody until the twelfth day or later.

TESTS FOR ANTIBODY FOLLOWING TRANSFER OF WASHED LYMPH NODE CELLS

Sensitization: *o*-Chlorobenzoyl Chloride

Exp. No.	*Method*	*Transfer*	*Recipient*	*P*[a]	DAYS FOLLOWING TRANSFER									
					1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*19*
XX	i.c.	0.9 ml. i.v. & i.p.	1434 ♀	0	+±	++	++± (Dale)	++ str	++±		+++			
XXII	i.m.	0.45 ml. i.v. & i.p.	1829 ♀	0			+		++				+±	±

NOTE: Antibody determined on small serum samples, tested by the Prausnitz-Küstner method described in the text.

[a] P = sample of serum taken before the injection of cells.

The gradual acquisition of antibody following cellular transfer could reflect a synthetic function of living cells or perchance be the result of extraction *in vivo* of antibody contained within the cells, or the result of the extraction of some "intermediate antigen," in the sense of Topley (17), that is able to engender antibody-formation with extreme rapidity.

Actually, with attention once centered on lymphoid cells as causally related to the formation of antibody, various attempts at direct extraction of the lymphocytes of rabbits have led to positive results (cf. Chapter 2), although usually of a disappointingly low order of concentrations. Such results have not yet been duplicated with guinea pig cells in the author's laboratory, probably because of difficulties attending the detection of very minute amounts of antibody. Upon injection into normal recipients, however, the issue is quite different, for the same cells give rise to appreciable levels of antibody. I am privileged to be able to report, prior to publication, that Dr. T. N. Harris,[4] pursuing in rabbits experiments similar to those shown here with the guinea pig, has found likewise that normal rabbits injected with cells yield far more antibody than can be obtained from the cells directly. Even with some initial content of antibody taken for granted, the source of the large extra amount of antibody appearing *in vivo* remains as a challenge.

As regards the idea of Topley, mentioned above, it can only be said that the same cell-mass would have to contain both the small amounts of antibody obtained upon grinding rabbit lymphoid cells directly and also the hypothetical intermediate antigen, and furthermore that this antigen must not only possess the specificity of the stable antigen originally injected but must itself be extraordinarily labile, since cells that have simply been frozen do not give rise to the new increment of antibody.

Apart from attempts at extraction, evidence linking antibody and cells intimately has been provided by two independently conceived approaches. First, Walzer and Glazer (19) have reported that the washed white cells from the blood of human beings who are subject to asthmatic attack and are possessors of circulating reagin give rise, upon deposition in the skin of a normal man, to local "flare-and-wheal" reactions when the corresponding allergenic extract is injected seven days later. In the second instance, analogous experiments being pursued in the author's

[4] An abstract has appeared (18) since the date of this symposium; see also more recent reports (37, 38, 39).

laboratory with animals indicate that, after cells are deposited in normal skin, local reactions can be obtained at the site of injection provided testing is postponed for approximately the same time as is requisite for the detection of circulating antibody when larger aliquots of the same cell suspensions are injected intravenously. Positive results, however, are not yet to be secured with regularity, and the cell-donors must be exquisitely hypersensitive. Two examples will suffice. It should be pointed out, apropos of Dr. McMaster's remarks on the accumulation of antibody at sites of injury, that local injury is equal at all test sites on the animals, yet only certain of the sites react.

Case 8. Evidence for the occurrence of cutaneous reactions of the evanescent-type at sites where washed cells have been injected intradermally. (*a*) *Lymph node cells.* Four days after the intracutaneous injection of 50 percent suspension of washed cells obtained from lymph nodes as in Case 5b, and of similar suspensions of cells secured from lymph nodes of normal animals and of animals (deliberately) inadequately sensitized, a mixture of picrylated casein as antigen and Pontamine Sky Blue 6B (cf. 16) was injected intravenously. (The dye was employed as indicator of capillary damage.) There was an immediate appearance of blue punctate dots just outside the area of deposition of cells secured from the lymph nodes of the well-sensitized guinea pigs. Three minutes later, the ingress of blue dye had resulted in a partial collar, horse-shoe in shape, applied around the anterior margin of this one site only, and with the lapse of 10 minutes the area had broadened and was intensely blue.

(*b*) *Splenic cells.* Three spleens of animals of exquisite sensitivity, prepared as in Case 5a, were employed to obtain three corresponding 50 percent suspensions of washed cells; several spleens from normal animals were pooled and cells secured in like manner. These four suspensions were injected into the skin of each of several normal guinea pigs. After the lapse of four days, picrylated casein (but without admixture of the dye employed in Case 7a) was injected intravenously. Positive reactions, of varying magnitude, developed at each of the three sites prepared with cells from sensitized animals, although the recipients did not react equally.

In summary, then, we have seen that cells capable of transferring hypersensitivities of the so-called "delayed type" and cells capable of giving rise to circulating antibody upon residence *in vivo* are obtainable from the same source: lymph nodes. When lymph nodes are teased out under fluid, and the freely separating cells are collected, small lymphocytes are preponderant to the extent of 95 percent or more, among the 70 to 80 percent of viable cells in our preparations. The weight to be

accorded our observations indicating that injured cells are ineffectual in producing either sort of effect is moot. If the activity is to be ascribed to the small and variable proportion of viable mononuclear cells that are not lymphocytes, one must conclude that these are exceedingly active, and that with proper selection of the cellular elements the same volume of cells as now employed may give a 20-fold to 50-fold increase in observed antibody concentrations.

The question arises, therefore, whether production of the sorts of dermal sensitivities under study here is the same as the production of circulating antibody; this solution would enjoy the distinction of being both neat and useful. While the results of future experimentation cannot be predicted, such a conclusion does not now appear justifiable for reasons that will not be detailed here. An alternate concept would be the existence of a plurality of immunological functions, all capabilities of the same stem cells. The particular effect observed following the transfer of cells would then be determined by the mode of treatment that the actively sensitized donor individuals had undergone. If this be correct, evidently both sorts of effect will be produced in certain instances, and in others perhaps only one of them.

In what way should progress be expected? The function of transferred cells may be sought by continuing extraction experiments. Attempts to transfer sensitivities of the delayed type by means of soluble materials have been reportedly successful in several instances, namely with blister fluids[5] secured in man by use of the Urbach-Koenigstein technique (cf. also Ballestero and Mom, 20), and with extracts of cavy splenic cells by Crepea and Cooke (21). Confirmation of these is still wanting, and the claims stand isolated. Apart from these reports, the work of Favour and his associates (4, 22–27) occupies an outstanding position in the preparation of extracts that presumably have to do with sensitivities of the delayed type. Miller and Favour (27) have actually been able to obtain from lymphocytes of the tuberculous subject—themselves subject to breakdown in the presence of tuberculin and complement—extracts that lead in turn to the breakdown of normal lymphocytes when tuberculin, complement, and extract are provided together.[6]

[5] It should be noted that blister fluids are often transferred without preliminary removal of cells, a possible source of error in interpretation of results.

[6] Certain other reports, dealing with neutrophilic leukocytes and erythrocytes, should be mentioned here, although in view of experimental results of the writer

The active component in the extracts, precipitating along with euglobulins of the plasma employed in preparing the extracts, possesses properties identical with those of the "plasma factor" discovered in the serum of certain tuberculous individuals by Favour and his colleagues. Both the lymphocytic extract and "plasma factor" lead to fairly rapid destruction of normal lymphocytes (within one hour). And by hindsight, the results of the curious experiments conducted by McJunkin (33) in 1921 might possibly be related to the preparation of extracts, of low but real potency, made from the tuberculous exudates of infected guinea pigs.

Possibly no one suggestion may exceed any other in worth, but, unless we are here dealing with a masked form of antibody contained in the cells, it does not appear improbable to the writer that we are handling, albeit in a mixed system, an enzymic mechanism that functions in the formation of antibody. In any event, progress in the understanding of antibody formation should lie ahead.

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it is hardly likely that either of these cells can effect transfer of dermal sensitivity. Furthermore, in view of the absence of these cell types among the cells freed from the lymph nodes certainly neither one is essential for mediating the process by which circulating antibody develops after cell transfer.

In man and guinea pig there has been found, besides lympholysis, some decrease in neutrophilic polymorphonuclear leukocytes when specific antigen is mixed with whole blood (22), although evidences of such an effect are often wanting (27, cf. 34). In the course of investigation of a different sort of allergy, but conducting a somewhat parallel experiment, Squier and Lee (28) reported that when ragweed extract was mixed with heparinized whole blood of patients markedly sensitive to this allergenic material there occurred a marked decrease—up to 43 percent—in polymorphonuclear leukocytes, and a minor decrease in lymphocytes; Squier (29) had previously pointed to "increased fragility" of white cells when allergic patients consumed the offending food. Again it has been shown that a large proportion of erythrocytes undergo lysis *in vivo* when small amounts of tuberculin are injected intravenously into the tuberculous rabbit (Sandage, *et al.*, 31, 32). This circumstance is reminiscent of a test that has been designed specifically to assist in detecting the presence of antibodies directed against components of the tubercle bacillus, the reacting system, held at 37° C., here consisting of normal erythrocytes coated with components of Old Tuberculin, complement, and antibody (33). Sandage and Birkeland (30, 32), indeed, are inclined to attribute their result to the stimulation of tuberculous foci.

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DISCUSSION

DR. JANEWAY: So far as is known the usual fate of normal lymphocytes in man is to survive a very few hours. It seems to be the cell with the most rapid turnover, although it undeniably remains morphologically intact in stored blood. *In vivo* it is well known that these cells invade the mucosa of the intestinal tract and other organs. Does Dr. Chase mean to imply that the transferred lymphocytes persist for a prolonged period in the skin and continue to form antibody?

DR. CHASE: I do not myself belong to the school of lympho-maniacs. I can only describe the experiments we have carried out. Whether or not transferred lymphocytes survive *in vivo* I do not know, but these cells are certainly durable in the icebox for several days, as tested with trypan blue in the presence of added serum. The latter fact may not, of course, have much bearing on the situation here dealt with. In the case of cells injected into the skin, however, it might not be necessary for them to survive for very long and yet initiate some process not yet understood. Since there is evidence for accumulation of antibody far beyond the time that transferred cells could be expected to survive, it seems to me that the life of the lymphocyte *in vivo* is not so important a consideration at the moment as auxiliary problems.

Chapter 11

FATE OF I^{131}-LABELED BOVINE GAMMA GLOBULIN IN RABBITS[1]

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The technique of labeling and tracing protein antigens and antibodies with radioactive iodine is being used increasingly in immunologic studies. Mention of a few of the more important chemical, physiological, and immunological features of this technique will indicate both its possibilities and its limitations. I^{131} can be added to most proteins in traceable amounts without altering the proteins immunologically. Accurate quantitation of I^{131} in blood, tissues, or immunologic precipitates is possible with a Geiger counter, using proper absorption and coincidence corrections. These radioactivity measurements indicate the concentration of labeled protein in the material tested. In addition, approximate histologic localization of the I^{131} protein can be obtained with autoradiographs, in which ordinary histologic sections are mounted on special photographic emulsions and, in effect, take a picture of their own radioactivity. Autoradiographs do not, however, permit the detailed histologic localization possible with fluorescent antibody (1) or dye-labeled proteins (2 and 3).

Protein antigens labeled with I^{131} appear to retain the I^{131} label *in vitro* (4) and *in vivo* (5) as long as they are immunologically active. The studies of Knox and Endicott (5), as well as our own observations, show close agreement between quantitative precipitin and radioactivity determinations of antigen.

Catabolism of I^{131} protein antigens liberates non-protein-bound I^{131}, and in animals prefed ordinary iodine, this liberated I^{131} is rapidly eliminated, primarily in the urine, with lesser amounts in breath and feces. Following injection of an I^{131} antigen, the radioactivity in blood and tissues can be separated into protein-bound and non-protein-bound

[1] This work was supported by grants from the Atomic Energy Commission and the United States Public Health Service.

fractions; the protein-bound activity represents residual antigen while the non-protein-bound I^{131} present in the blood and tissues, plus that excreted in the urine, provides a reliable index of the rate of antigen catabolism. This technique, therefore, gives an excellent quantitative measure of antigen concentration in blood and tissues and also indicates rate of antigen catabolism.

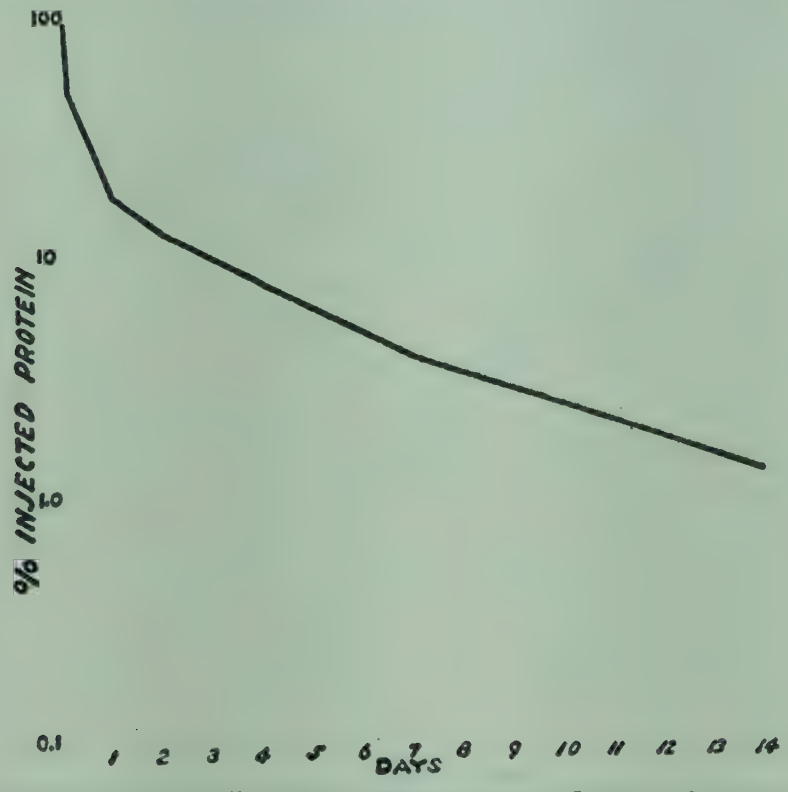

FIGURE 1. BLOOD LEVELS OF HOMOLOGOUS GAMMA GLOBULIN

Before discussing our observations on the rabbit's handling of the antigen, I^{131}-labeled bovine gamma globulin (I*BGG), let us first look at the rabbit's metabolism of his own plasma globulin. Madden and Whipple (6) have shown that plasma proteins exist in dynamic equilibrium in vascular and extravascular fluids and serve to supply, in part, the nutritional needs of the body's tissues. Our findings using homologous gamma globulin labeled with I^{131} (I*RGG) concurred with theirs. Figure 1, which is a semilogarithmic plot of the labeled gamma globulin content of blood against time in days, shows that within one day after intravenous injection there was a sharp drop in the I*RGG blood content to nearly one third the amount injected. This drop represents the equi-

libration of I*RGG between vascular and extravascular components of the plasma protein pool and indicates somewhat more gamma globulin in extravascular than in vascular fluid. Once the equilibrium was established, the I*RGG disappeared at a constant rate with a half-life of about 4.7 days, representing the rate of catabolism of homologous gamma globulin. This rate of I*RGG catabolism agrees almost exactly with the rate of breakdown of passively administered homologous antibody in the rabbit (7, 8, 9).

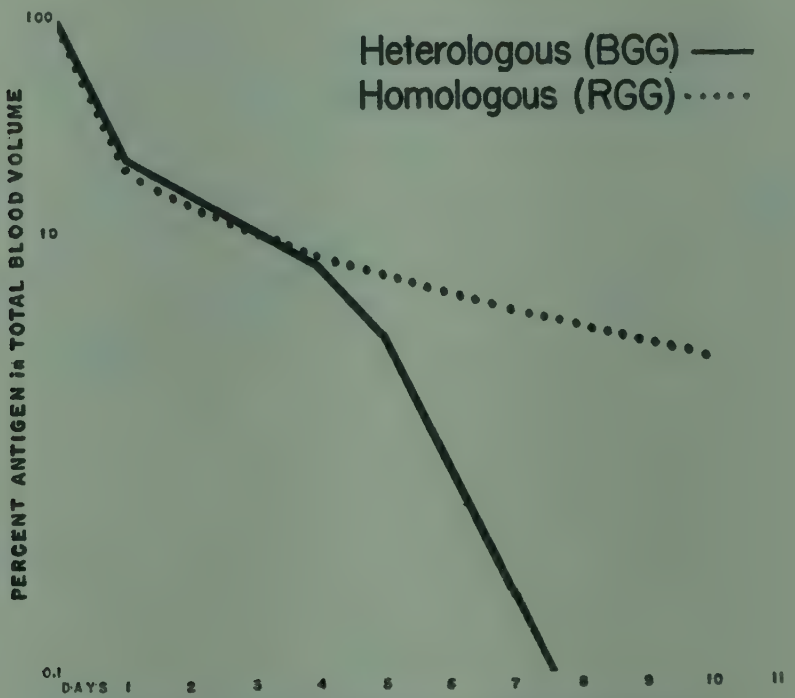

FIGURE 2. BLOOD LEVELS OF GAMMA GLOBULINS

I*BGG injected intravenously into rabbits in doses varying from 1 mg. to 500 mg. was handled similarly to the I*RGG for the first four days (10). The second graph of blood content of labeled protein again shows the disappearance curve for homologous gamma globulin. In addition, the solid line represents the amount of I*BGG in the blood following a 75 mg. injection and shows an equilibration period followed by a three-day period of catabolism similar to that for the homologous protein. It appears that this antigenic, heterologous protein equilibrates between vascular and extravascular fluids just as does homologous protein. In so doing, the antigen, no doubt, comes into intimate contact with most or all of the body's tissues. The catabolism of heterologous gamma

globulin at a rate almost identical with that of homologous gamma globulin for four days would further suggest that in this initial period the body may utilize the heterologous globulin similarly to its own globulin.

After the fourth day, the disappearance rate of I*BGG from the blood increased sharply. It is most likely that this increased elimination rate is caused by the appearance of antibody which, however, could not be detected by our complement-fixation technique (11)[2] until all antigen had been eliminated, seven days following injection. The first antibody to appear probably combines with antigen in extravascular or intravascular fluids and the combination is rapidly eliminated from the circulating fluids and catabolized, as will be discussed later. Since this is accomplished without measurable accumulation of antigen in any organ or tissue, it is evident that the breakdown of antigen is either an extremely rapid process or one widespread throughout the body, or both.

The elimination of I*BGG from the various tissues approximately paralleled its elimination from blood, as would be expected with a dynamic equilibrium of the antigen in the body. In Figure 3 the amount of radioactivity per gram of tissue or blood following an intravenous injection of 75 mg. I*BGG is plotted against time in days. The activity concentration was higher in blood than in any tissue, and disappearance rates were, in general, parallel with a slight lag in the disappearance of radioactivity from the tissues after the fourth day. All radioactivity levels were insignificant after the seventh day, with no tissue showing measurable retention of antigen. Complement-fixation tests for antigen in the tissues after the seventh day were also negative.

Detectable circulating antibody appeared on the seventh day after virtually all antigen had disappeared. This is in keeping with the findings of Hawn and Janeway, who reported disappearance of BGG and appearance of antibody between one and two weeks following injection of 2 gm. of antigen (12). Following injection of 75 mg. of I*BGG the peak serum antibody concentration was reached in ten days, three days after the elimination of all detectable antigen as shown in Figure 4. Antibody

[2] All antibody determinations were done by the complement-fixation technique described by Kent, *et al.* This method employs a dilution of antigen (1:500,000) which is optimal for the detection of minimal amounts of antibody with four 50 percent hemolytic units of complement. The minimal amounts of antibody as detected are of the order of 10 mgm. antibody nitrogen per c.c. of serum.

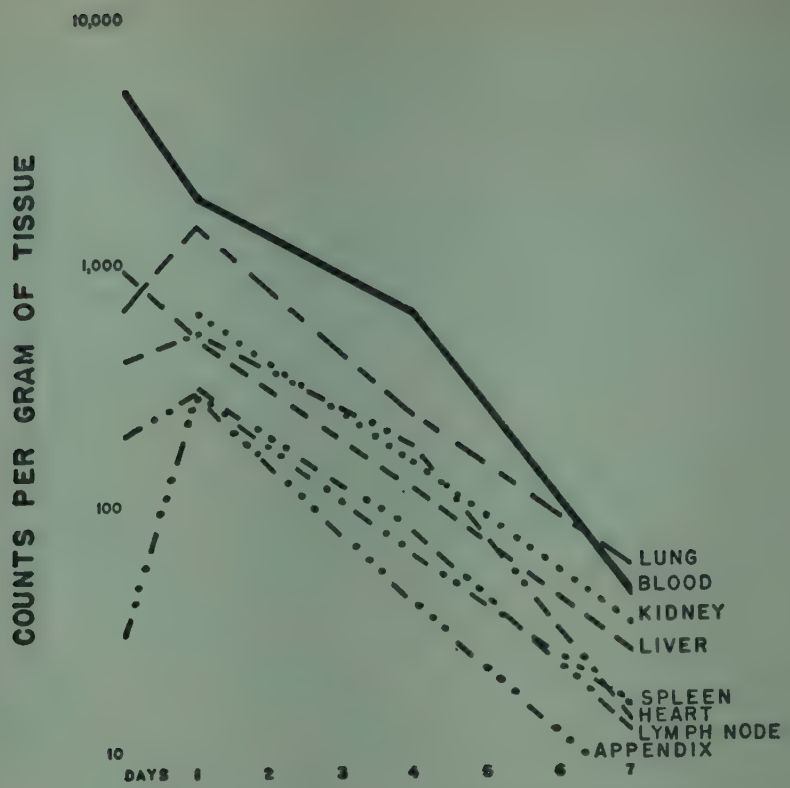

FIGURE 3. TISSUE ANTIGEN CONTENT I*BGG

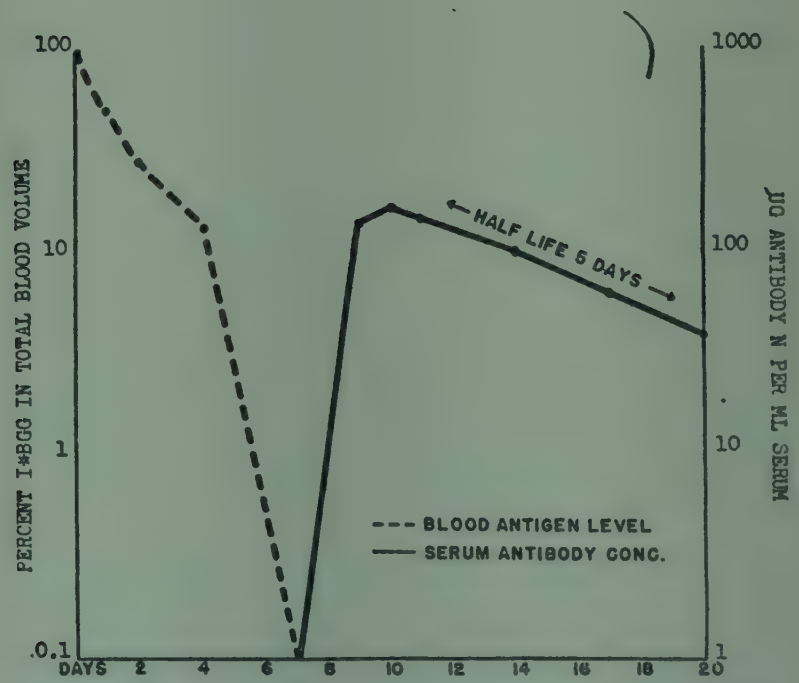

FIGURE 4. I*BGG ELIMINATION AND ANTIBODY RESPONSE

concentrations for the next ten days as indicated by quantitative precipitin determinations decreased at a logarithmic rate with a half-life of five days. This rate of decline of antibody levels was approximately the same as the rate of elimination of passively administered I*RGG or homologous antibody (7, 8, 9) and indicated little antibody production during this period.

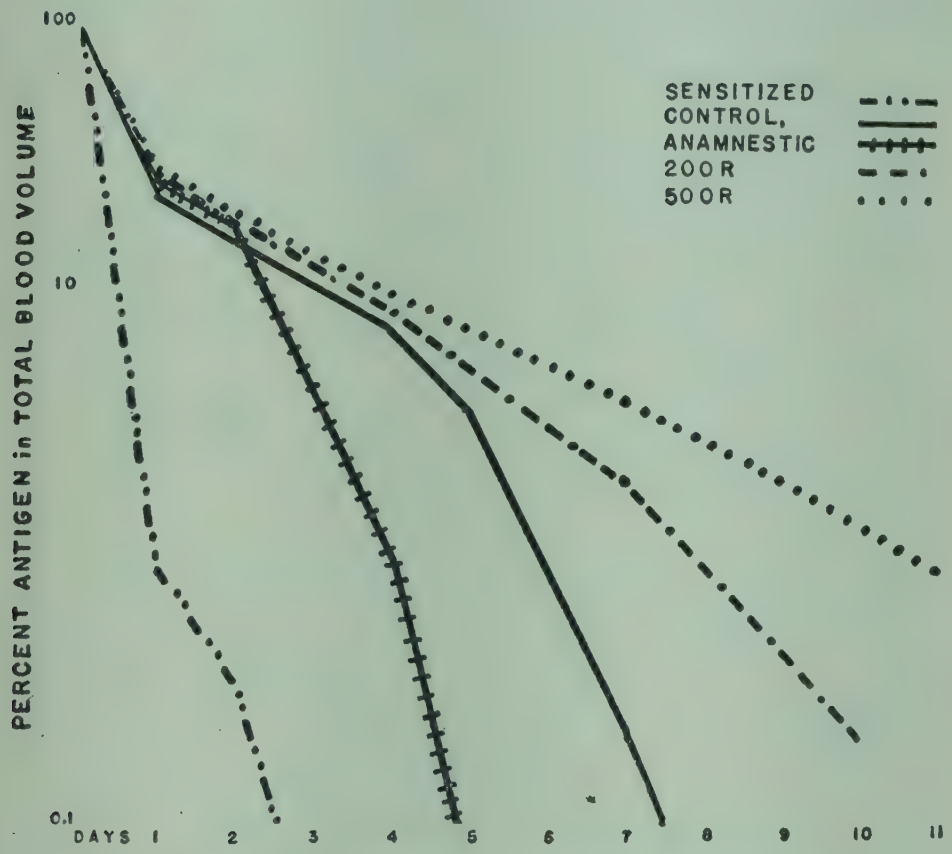

FIGURE 5. SENSITIZED AND X-RAYED ANIMALS

We found considerable support for the view that the increased antigen disappearance rate seen after four days in I*BGG-injected control rabbits is caused by appearance of antibody. Figure 5 compares blood antigen levels in five experimental groups of rabbits, each with a different immune response: actively sensitized rabbits with detectable circulating antibody present at the time of injection of antigen showed rapid disappearance of antigen immediately; previously sensitized animals without detectable circulating antibody showed a rapid antigen disappearance beginning two days after injection, in keeping with an accel-

erated, anamnestic response; animals receiving 200 r whole body x-radiation showed delay in and decrease of rapid antigen elimination, corresponding to the inhibition of the immune response resulting from the radiation; animals receiving 500 r x-radiation showed neither rapid antigen elimination nor any antibody response. The appearance of detectable serum antibody in these five groups of rabbits always followed immediately upon the rapid disappearance of antigen, as seen in Figure 6. Here the five antigen disappearance curves are given in temporal

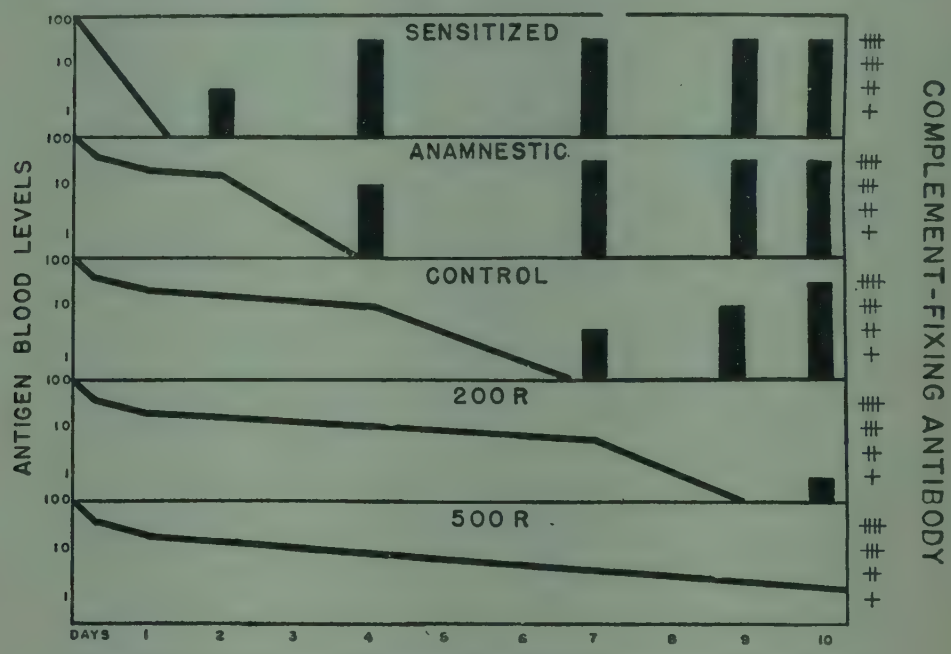

FIGURE 6. ANTIGEN ELIMINATION AND ANTIBODY RESPONSE

relation to the appearance of complement-fixing antibody, indicated by the black bars. In the first four instances, rapid antigen disappearance was closely followed by appearance of detectable antibody, while after 500 r neither rapid antigen elimination nor detectable antibody was seen.

Further support for the relationship of antibody to rapid elimination of antigen was found after passive administration of antibody to rabbits with circulating I*BGG. In Figure 7 the I*BGG concentration in blood is plotted against an expanded time scale. The injection of antibody, 100 mg. on second day and 40 mg. on third day, caused a prompt drop in blood antigen content. The rise in blood antigen level shortly after the first injection represents the reestablishment of equilibrium of anti-

gen between the depleted vascular fluid and the antigen-containing extravascular fluid.

On the basis of these interrelationships between antigen disappearance rate (ADR) and appearance of antibody, we have used the mathematical expression of ADR as an index of immune response (13). This measure is extremely sensitive and will reveal an immune response which may produce only enough antibody to combine with the antigen present,

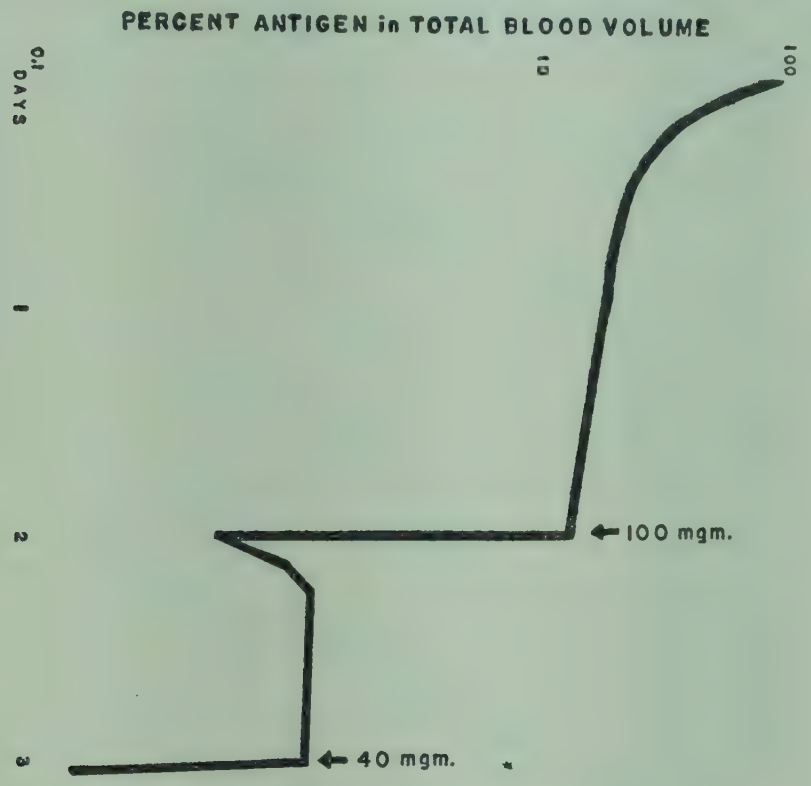

FIGURE 7. INJECTION OF ANTIBODY

and not enough to cause a detectable excess in the serum. Such weak antibody responses may occur after moderate doses of X-ray or following extremely small doses of antigen and result in rapid antigen elimination without detectable antibody. Later demonstration of a specific anamnestic response indicates that such animals have made a previous immune response to the given antigen.

The mechanism of rapid elimination of the antigen-antibody combination from the blood and the associated rapid antigen catabolism are not completely understood. It is most likely that antigen-antibody complexes would be taken up by phagocytic cells, thus accounting for the

rapid removal of antigen from the blood. In investigating antigen catabolism, we attempted to find whether the increased rate of catabolism in sensitized animals could be accounted for by presence of antibody alone and, therefore, presumably by increased phagocytosis, or whether there

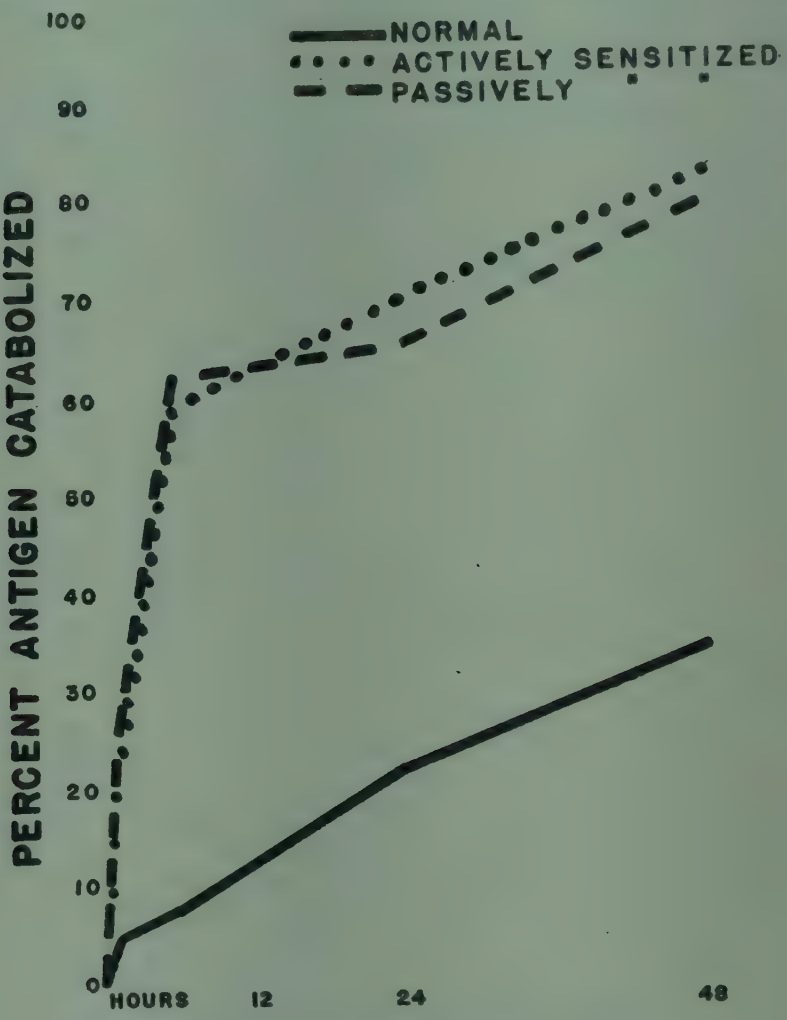

FIGURE 8. ANTIGEN (I*BGG) CATABOLISM

was also a cellular enzymatic adaptation associated with the process of sensitization, which facilitated more rapid antigen catabolism (14). To do this, we compared the rates of I*BGG catabolism in actively sensitized rabbits and in passively sensitized rabbits given 95 mg. of antibody, an amount calculated to be approximately equal to the amount in those actively sensitized. Figure 8 shows the rates of I*BGG catabolism, as measured by non-protein-bound I^{131} in urine, blood, and tissues in

actively sensitized, passively sensitized, and control rabbits for the first 48 hours after intravenous injection. The rates of antigen catabolism for actively and passively sensitized rabbits were almost identical. The antigen disappearance rates in actively and passively sensitized groups were also similar. It appears, therefore, that both the increased rate of disappearance of I*BGG from the blood and the rapid catabolism found in sensitized animals can be accounted for by the presence of

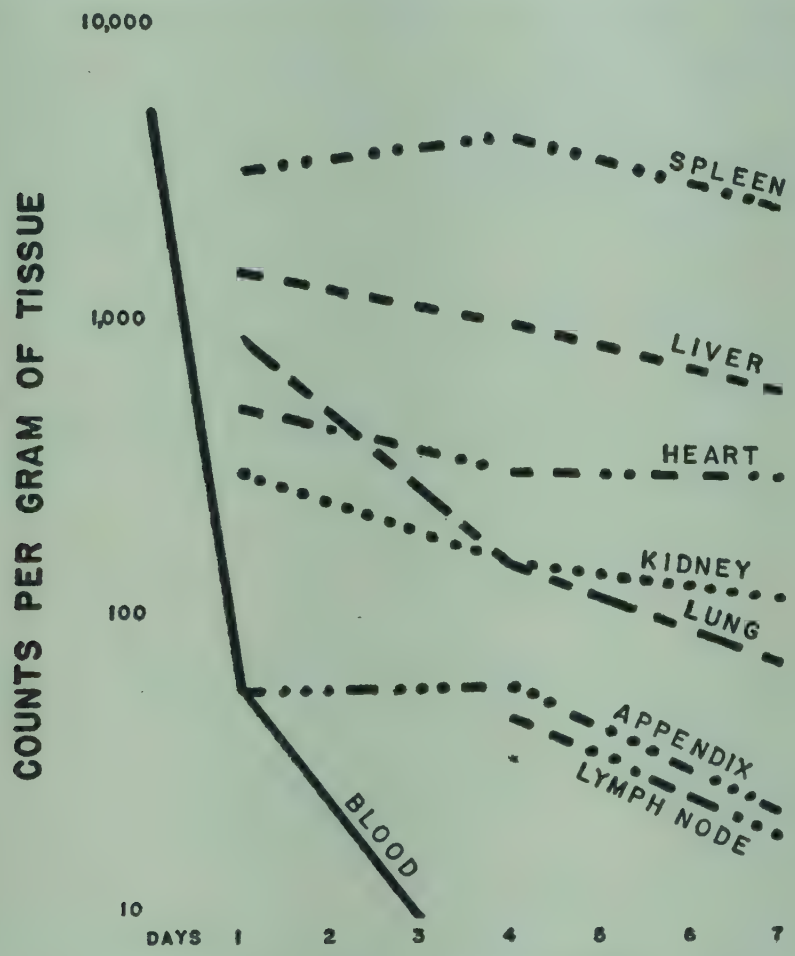

FIGURE 9. TISSUE ANTIGEN CONTENT I*BGG-R SALT

antibody alone. Whether the sensitized animal makes any physiologic or enzymatic adaptation to an antigen such as I*BGG other than the production of specific antibody cannot be definitely stated at this time. However, there is nothing that happens in the immune catabolism of I*BGG that cannot be duplicated in a control animal by passive sensitization.

Our observations on antigen localization agree with the antigen tracer study of human gamma globulin using the fluorescent antibody technique (1). There is, however, a marked discrepancy between our findings and those obtained by workers using BGG or similar proteins labeled with various azo dyes (3). The azo dye-labeled proteins were found concentrated and retained for long periods of time in the reticulo-endothelial system. In an effort to explain these differences, we attached to I*BGG by azo conjugation one of two compounds, R salt (15 and 16) or para aminobenzoic acid, and traced these doubly labeled antigens by both color and radioactivity. Using these antigens, we found an uptake and retention of antigen by the reticulo-endothelial system, just as described by those using the various azo dye-labeled proteins. Figure 9 indicates azo-I*BGG concentration in blood and tissues following intravenous injection of 75 mg. of the antigen into control rabbits. The blood level fell rapidly while tissue concentrations, especially in those tissues rich in reticulo-endothelial cells, dropped very slowly. The tissue retention of azo-I*BGG is in marked contrast to the findings with I*BGG where no tissue antigen retention occurred (Fig. 3). Thus, it appears that azo labeling markedly changes the *in vivo* behavior of protein antigens such as BGG.

SUMMARY

1. Tracing of the antigen I*BGG in rabbits indicates that the antigen is handled much like the animal's own gamma globulin until antibodies to it are formed. Before the appearance of antibodies, the antigen exists in equilibrium between vascular and extravascular fluids, has free access to the tissues of the body, and is neither concentrated nor retained by any tissue or organ.

2. With the appearance of antibody, the antigen is rapidly removed from blood and extravascular fluids, probably by phagocytic cells, and is rapidly catabolized.

3. There does not appear to be any cellular enzymatic adaptation necessary for the sensitized, rapid catabolism of antigen.

4. The appearance of antibody is first reflected in the increased antigen disappearance rate. The maximum serum antibody concentration is reached about three days after elimination of all detectable antigen. During the following ten days the antibody level drops at a rate almost

identical to the rate at which passively administered antibody is lost, indicating that little antibody production persists after peak antibody concentration is reached.

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Chapter 12

FATE AND EFFECTS OF INJECTED PROTEIN ANTIGENS[1]

By Charles A. Janeway, *Harvard Medical School, and the Children's Medical Center, Boston*

THE PROBLEM OF SERUM DISEASE

The pathogenesis of human serum disease has interested numerous investigators since von Pirquet and Schick described the disease and suggested its dependence upon the reaction of the injected foreign protein with the antibody formed against it, in their classical monograph, published in Vienna in 1905 and now happily translated and reprinted in this country (1). They pointed out that the symptoms of the disease were not due to the precipitins, but to what, for lack of available methods of isolation and characterization, they called "vital antibodies."

In 1918, during the period when unrefined horse antiserum was being tried in large amounts for the treatment of pneumococcal and meningococcal infections, Longcope and Rackemann (2) showed that the symptoms of the disease developed at a time when the amount of horse serum present in the blood was diminishing rapidly and when precipitins for horse serum were beginning to appear. We now know that they were working with a solution containing multiple antigens, so that exact interpretation of the relationship between the interaction of antigen and antibody in the body and the symptoms of the disease was very difficult.

[1] The work described in this paper has been supported, to a large extent, by grants from the National Institutes of Health, U.S. Public Health Service. These studies have been carried out with a group of associates, whose thought and efforts represent the major share of any contribution which our laboratory has been able to make to this problem. These colleagues are Dr. James T. Heyl, now physician to Phillips Exeter Academy; Dr. Clinton van Zandt Hawn, Pathologist, the Mary Imogene Bassett Hospital, Cooperstown, N.Y.; Dr. Frederick C. Moll, now of the University of Washington; Dr. Louis Schwab; Dr. Ralph J. P. Wedgwood; Dr. David Gitlin; Dr. Harrison Latta; Dr. Thomas C. Hall; Dr. Marjorie Kirk; Dr. Henry Brean; and our able technical assistants, Misses Ladislas Wojcik, Ann Shwachman, Katherine Marx and Miriam Ekdahl.

Much of the material in this chapter was first presented as the Annual Schick Lecture at the Mount Sinai Hospital, New York City, on March 29, 1950.

During the first part of the war, we had the opportunity to observe an epidemic of serum sickness resulting from the injection into human volunteers of large doses of a highly purified foreign serum protein, as part of a study of its possible value as a plasma substitute (3). This crystallized bovine serum albumin, which was 99.9 percent pure, came close to being a single antigen. The delayed reactions, which developed in certain of the volunteers two to three weeks after injection of a dose of 25 grams, manifested themselves with symptoms which were fairly uniform in distribution, but varied greatly in severity. The correlation, which we had expected since we were presumably dealing with a single antigen, between the subsidence of symptoms, the disappearance of bovine albumin from the serum, and the appearance of antibodies, as determined either by the precipitin test or the skin test, was observed in only a few of the severe cases. No satisfactory explanation for these observations has yet been found: they indicate some of the complexities of the problem of serum disease in man, even when it results from the injection of a very pure protein preparation.

Karelitz and his associates (4) have recently demonstrated that the antibody responsible for serum sickness in humans given equine antitoxin is apparently distinct from precipitating antibody, but similar to skin-sensitizing antibody, thus confirming von Pirquet and Schick's hypothesis. Unfortunately this work was done with a mixed antigen.

Further suggestions bearing on these possible distinctions may be gleaned from the differences between the skin-sensitizing and blocking antibodies described by the allergists and from the observation of Campbell (5) that the "reagin" or skin-sensitizing antibody in ragweed-sensitive individuals is concentrated in the alpha and beta globulin fraction and from Vaughan's (6) demonstration that it is found in Fraction III, rather than in Fraction II, in which the gamma globulins with antibody activity toward such protein antigens as viruses and bacterial toxins are found.

The study of serum sickness took on new importance when Rich (7) reported the finding of diffuse inflammatory lesions of the arterial system in patients dying during the course of delayed reactions due to foreign serum or to sulfonamides. Although similar observations had been previously made by others studying reactions to foreign proteins, the subsequent demonstrations that similar lesions could be produced in rabbits by large injections of horse serum (8) has led to a great deal of experi-

mental work, spurred on by the provocative analogies drawn by Rich (9) between these lesions of experimental hypersensitivity in the rabbit and the lesions of human rheumatic fever, glomerulonephritis, periarteritis nodosa, and disseminated lupus erythematosus. With evidence that the development of anaphylactic hypersensitivity was accompanied by morphological changes, the problem of serum disease assumed added significance. The basic question for the student of disease is "what is the pathogenesis of these tissue lesions?" Our group has undertaken to study this question during the past few years, as have many others; the remainder of this paper will attempt to summarize these studies.

FATE OF INJECTED PROTEIN ANTIGENS

Since Rich's observations had been made with horse serum, containing a mixture of antigens for the rabbit, an initial comparison of the behavior of two relatively pure chemically and immunologically distinct heterologous plasma proteins was made (10). After satisfying ourselves that bovine serum would produce lesions identical with those described by Rich following the injection of horse serum, the effects of single large injections (1 gram/kilo) of crystallized bovine serum albumin and bovine serum gamma globulin[2] were compared. Not only were the lesions produced found to be quite different, but the time intervals between injection and the development of lesions as well as the disappearance rates of the two proteins from the circulation contrasted sharply. These differences are indicated in Table 1.

Working on the hypothesis that the lesions were the result of the interaction between antigen fixed in the tissues and antibody either developing locally or brought from sites of production in other parts of the body, our attention was turned to methods for study of the tissue distribution of foreign proteins, in an effort to demonstrate the localization of these antigens in those sites where lesions subsequently appeared.

Kay (11, 12), in a series of beautiful experiments on nephrotoxic nephritis in the rabbit, had shown that the nephritis, which developed eight or nine days after injection of duck anti-rabbit kidney serum, was presumably due to the fixation of duck serum in the rabbit kidney and its subsequent interaction with antibody to duck serum as it developed

[2] Both albumin and globulin were kindly supplied by Drs. Jules Porsche, John Glynn, and James P. Lesh of the Armour Laboratories, Chicago.

TABLE 1

COMPARISON OF RESPONSE OF RABBITS TO INTRAVENOUS INJECTION OF 1 GRAM PER KILOGRAM OF DIFFERENT BOVINE SERUM PROTEINS

	SEROLOGIC CHANGES		PATHOLOGIC CHANGES		
Antigen	*Percent Developing Antibodies*	*Interval (Days) Before Appearance of Antibodies*	*Percent Developing Lesions*	*Time (Days After Injection) When Acute Lesions Found*	*Location of Principal Lesions*
Bovine serum	88	14–28	100	14–20	Arteries
Crystallized bovine serum albumin	20	14–28	47	14–20	Arteries
Bovine serum gamma globulin	100	7–14	80	7–12	Liver Kidney Heart Joints

in the injected animal. In this system the antigen was localized by virtue of its immunological specificity. Dr. Pressman's studies of radioactively tagged organ-specific antisera have substantiated Dr. Kay's conclusions (13). The only indicator for fixation of antigen in Kay's and our experiments, however, was the pathological finding of lesions presumed to have an immunologic basis.

Accordingly, the use of labeled antigens was investigated. Many studies of the distribution of foreign proteins labeled with azo dyes have been made in the past. Recent work by Gitlin (14) had indicated that in the mouse the distribution of the dye-labeled protein depended both upon the dye to which a particular protein was coupled and upon the protein to which a particular dye was attached.

The visible localization of three different azo-dye-labeled human serum albumins[3] was studied after intravenous injection and compared with the localization of the dyes alone and of india ink (15). Dye granules were regularly found up to eight days after injection in animals given the azoproteins but not in those receiving the dyes alone. Certain rather striking differences in distribution between these azoproteins

[3] Evans blue-azo-human serum albumin, orange G-azo-benzidine-azo-human serum albumin, and acriflavine-azo-human serum albumin.

were observed, particularly the accumulation of dye in the glomeruli with acriflavine-azo-human serum albumin and in the cells of the proximal convoluted tubules with the other two azoproteins, but, in general, colored material was scattered throughout the macrophage system. "Granules were also found in Anitschkow 'myocytes' of the heart and in the epithelial cells of the liver, adrenal cortex, and proximal convoluted tubules of the kidney. In addition, fibroblasts and synovial cells appeared to contain granules." In such tissues as the myocardium, endocardium, synovial membranes, and adventitia of the arteries, colored granules were observed in relatively large amounts in sites where the lesions of experimental hypersensitivity are most prone to develop.

Although such dye-labeled proteins appeared to be very satisfactory tracers for the distribution of foreign protein, particularly in view of the evidence for stability of the azo linkage *in vivo*, there is some doubt concerning the extent to which these results can be applied to an interpretation of the behavior of unlabeled native proteins. Ultracentrifugal analysis of these azo-human serum albumin preparations demonstrated an increase in components sedimenting faster than the major albumin component, thus indicating molecular change and aggregation as a result of diazotization. Immunological evidence suggested a considerable degree of heterogeneity, as shown by the failure of antiserum against native human serum albumin to precipitate 42 percent of the color from solutions of two of the azoproteins, Evans blue-azo-human serum albumin and orange G-azo-benzidine-azo-human serum albumin, in the zone of antibody excess (16). This finding, confirming previous observations in Heidelberger's laboratory (17) that the most highly colored fraction of an azo-egg albumin would hardly precipitate with anti-egg albumin serum, helps to explain the differences in disappearance rates for these azo-proteins when their concentration in the serum, as estimated by color intensity, was compared to the concentration of azoprotein, as measured by precipitation with an antiserum to native human serum albumin. In every case values by the immunochemical method were higher than values obtained by the measurement of color intensity (Fig. 1). Presumably the most heavily conjugated molecules, principally detected by the second method, were removed more rapidly than the less conjugated molecules principally detected by the former method. This is consistent with the demonstration by Fine and Seligman (18) that the more heavily

they labeled the proteins of dog plasma with radio-bromine the more rapidly the labeled protein disappeared from the circulation.

On the other hand, studies with radio-iodine (I^{131} obtained from the Oak Ridge Laboratories) labeled human serum albumin and bovine serum gamma globulin prepared by Dr. Latta (19) indicated slight if any change in their behavior as compared to the native proteins.[4] Unfortunately, the specific activity of the labeled proteins, purposely kept

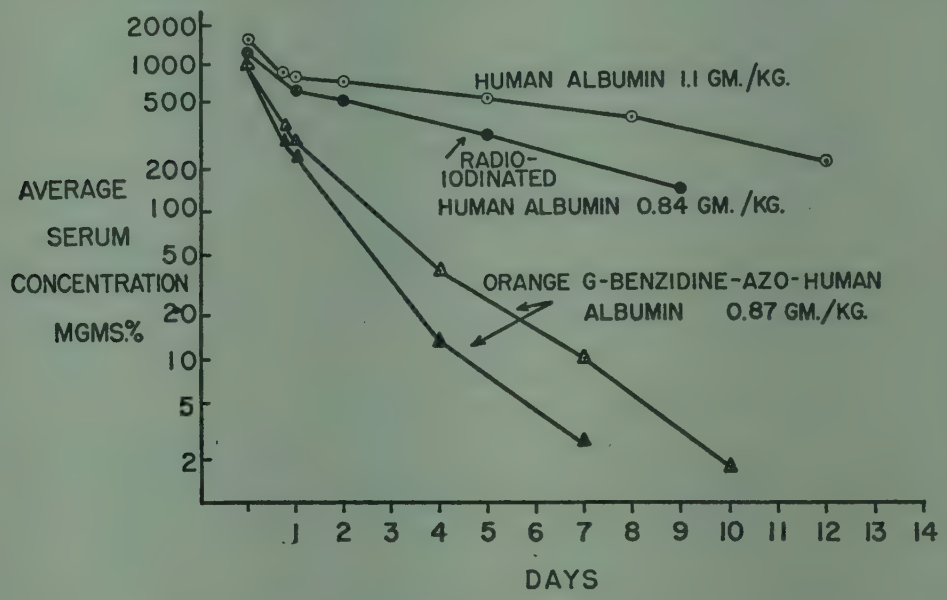

FIGURE 1. COMPARISON OF DISAPPEARANCE CURVES (RABBITS) FOR LABELED DERIVATIVES WITH THE CURVE FOR NATIVE CRYSTALLIZED HUMAN SERUM ALBUMIN

Measurement by immunochemical method with antiserum against native human serum albumin. Solid triangles indicate measurement by color intensity.

at a minimum to avoid the inhibitory effect of radiation on antibody formation, was insufficient to give accurate tissue localization by the radio autographic technique. However, the use of I^{131} as a label appears very promising. Counts of radioactivity in blood and tissues gave no evidence of concentration in any organ except the thyroid gland. Excretion of I^{131} in the urine indicated that the fall in the blood level was probably an indication of metabolism of the labeled protein. Table 2, in which the half-lives of some of the proteins studied in our laboratory

[4] Eisen and Keston (20) found that if bovine serum albumin was lightly labeled with I^{131}, it could not be distinguished immunologically from the native protein.

are given, points up the inadequacy of the azoproteins and the promising results with the I^{131} labeled proteins for studies of the distribution and localization of native proteins.

TABLE 2

AVERAGE HALF-LIFE OF REPRESENTATIVE NATIVE AND LABELED FOREIGN SERUM PROTEINS IN SERUM OF RABBITS AFTER INTRAVENOUS INJECTION[a]

Protein	*Number of Rabbits*	*Average Half-Life (Days)*[b]
Native Human Serum Albumin	5	5.5
Iodinated (I^{131}) Human Serum Albumin	3	4.5
Orange G-azo-benzidine-azo Human Serum Albumin	10	1.5
Evans blue azo-Human Serum Albumin	10	1.7
Acriflavin-Azo-Human Serum Albumin	7	2.2
Native Bovine Serum Albumin	5	5.1
Native Bovine Serum Gamma Globulin	5	1.9
Iodinated (I^{131}) Bovine Serum Gamma Globulin	3	1.8

Source: Modified from Table 1, Gitlin, Latta, Batchelor, and Janeway, *J. Immunol.*, 1951, 66:451–461.

[a] Blood levels determined by precipitation with specific antiserum to native protein and spectrophotometric analysis of the specific precipitate.

[b] Determined from rate of disappearance from serum between second and sixth days after injection (second phase of curve: see Fig. 3).

Other papers by members of this symposium (13, 21, 22, 23, 24) will indicate how well they have succeeded in extending the use of labels to study of the distribution and behavior of injected foreign proteins. Technical difficulties in the use of all labeled proteins, either antigens or antibodies, are considerable; it must be admitted that we have not succeeded in proving the postulated localization or persistence of crystallized bovine serum albumin or bovine serum gamma globulin in increased amounts in those sites where lesions develop in injected rabbits.

The disappearance curves for crystallized bovine serum albumin and bovine serum gamma globulin from the circulation of injected rabbits have three phases (Fig. 2). During the first 24 hours, there is a rapid fall in concentration, followed by a second phase, in which there is a

steady rate of disappearance for a period of several days (straight line when plotted logarithmically), and finally, in many instances, a third phase of accelerated decline in concentration. It seems probable that the first phase is due to equilibration of the protein between the circulating blood, the interstitial fluid, and the tissues. After equilibration takes place, the steady rate of removal characteristic of the second phase pre-

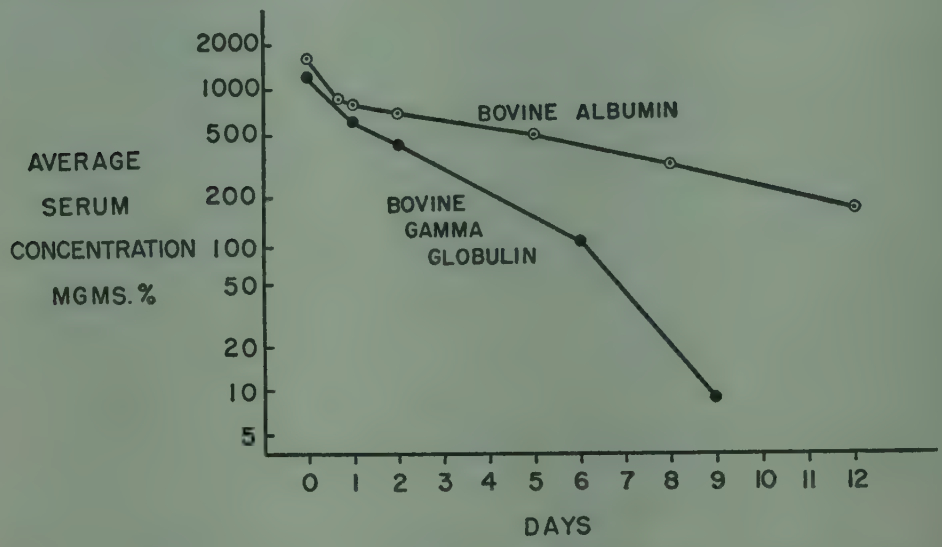

FIGURE 2. DISAPPEARANCE CURVES (RABBITS) FOR CRYSTALLIZED BOVINE SERUM ALBUMIN AND BOVINE SERUM GAMMA GLOBULIN, AS DETERMINED BY THE IMMUNOCHEMICAL METHOD
Dose of heterologous proteins was 1 gram per kilogram.

sumably indicates metabolism or storage of the foreign protein. The acceleration, which occurs in the third phase, is probably the result of interaction of the foreign protein with its antibody, thus rendering it non-reactive and probably hastening its removal.

EFFECTS OF INJECTED PROTEIN ANTIGENS

The serologic and pathologic responses to the injection of foreign serum proteins have been the subject of intensive study recently. Lesions such as those observed by Rich and Gregory and by our group have been studied particularly by Dammin, Bukantz, and their associates (25) and by Ehrich, Seifter, and Forman (26). The injection of 1 gram of crystallized bovine serum albumin per kilogram is followed in a certain percentage of rabbits by the development in about two weeks of pathologi-

cal changes in which segmental acute inflammatory lesions of the arteries predominate. Similar lesions can be produced by the injection of horse serum or bovine serum. An equal dose of bovine serum gamma globulin gives rise to lesions of the liver, endocardium, myocardium, synovial membranes, and glomeruli of the kidneys (10). The latter lesion, a proliferative type of glomerulonephritis, is most constant, occurring between 7 and 12 days after injection in about 80 percent of rabbits. The acute phase of this lesion coincides with the period when antigen is disappearing rapidly from the circulation. Following the disappearance of antigen and the appearance of antibody, which develops uniformly in rabbits given this dose of bovine serum gamma globulin, healing of the lesion takes place. This complicates efforts to correlate the development of antibody with the production of lesions, since lesions may not be detectable if the animals are sacrificed when antibody is readily demonstrable.

Because of the uniform response of rabbits to a dose of 1 gram of bovine serum gamma globulin per kilogram we have used this antigen and restricted our attention to the glomerular lesions in almost all our studies on the pathogenesis of lesions of hypersensitivity. More and Waugh (27) improved the technique for the production of renal lesions by using unilaterally nephrectomized rabbits and by the injection of a small desensitizing dose of antigen followed by a second large dose between one and two weeks after the initial injection. We have found this double injection technique useful in increasing the severity of the renal lesions, without nephrectomy. Although it simplifies the task of the pathologist, it complicates the interpretation of the serological responses.

The fact that the best time to sacrifice the animals for demonstration of acute renal lesions was at a time when antibody was not always demonstrable led us to investigate the behavior of serum complement, on the hypothesis that it might be fixed by the *in vivo* antigen-antibody reaction which we presumed was occurring. This hypothesis has proved to be correct, and a depression of the activity of hemolytic complement has always been observed at the time when antigen is disappearing rapidly, with a rapid return to normal or even slightly higher than normal levels when the antigen disappears and antibody becomes demonstrable (28; see also Fig. 3).

With these tools, the pathogenesis of the glomerular lesions has been

investigated. Kay (12) had previously demonstrated that the renal lesions developing in rabbits eight days after the injection of anti-kidney duck serum could be prevented by the administration of sufficiently heavy doses of X-radiation to prevent the formation of precipitins for duck serum. Bukantz, Dammin, and their associates (29) were able to prevent the development of vascular lesions, the Arthus phenomenon,

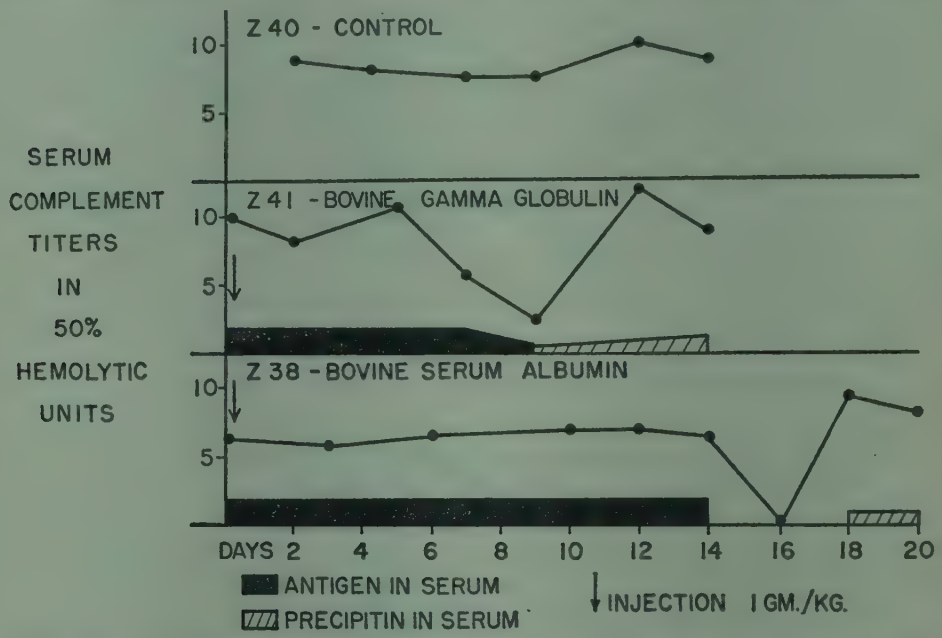
Z 40 - CONTROL
Z 41 - BOVINE GAMMA GLOBULIN
Z 38 - BOVINE SERUM ALBUMIN
SERUM COMPLEMENT TITERS IN 50% HEMOLYTIC UNITS
10
5
DAYS 2 4 6 8 10 12 14 16 18 20
ANTIGEN IN SERUM
PRECIPITIN IN SERUM
INJECTION 1 GM./KG.

FIGURE 3. CHARACTERISTIC BEHAVIOR OF LEVEL OF SERUM COMPLEMENT IN INDIVIDUAL RABBITS FOLLOWING INJECTION OF SINGLE LARGE DOSE OF HETEROLOGOUS SERUM PROTEIN
Control rabbit (Z40) is included to show minor fluctuations observed with repeated bleedings. Note sharp drop in complement titer at time antigen disappears from serum and its rise as free antibody appears in circulation. Also note characteristically different time sequences for bovine gamma globulin and for bovine albumin.

and the formation of precipitins in rabbits injected with horse serum by the administration of nitrogen mustards. Using X-radiation or nitrogen mustards (either methylbis [chloroethyl] amine hydrochloride or tris [chloroethyl] amine hydrochloride), administered in sufficient doses to the rabbits to maintain a marked leukopenia before injection of the antigen, Schwab and his associates were able to prevent the characteristic drop in serum complement, precipitin production and development of renal lesions following the injection of bovine serum gamma globulin

(28, Table 3). The inhibitory effect of X-radiation on horse serum-induced lesions in rabbits has been reported by others (30). These observations strongly suggest that the formation of antibodies is necessary for the development of lesions and for the drop in complement, and thus that both of these phenomena are the result of interaction of antigen and antibody. However, another possible interpretation of these results is that inflammatory lesions could not develop in leukopenic animals. Unfortunately no method of preventing antibody formation without depressing the activity of the bone marrow has been devised.

TABLE 3

EFFECT ON RESPONSE OF RABBITS TO INJECTION OF BOVINE SERUM GAMMA GLOBULIN (1 GRAM/KILO.) BY X-RADIATION AND NITROGEN MUSTARDS

Experimental Group	*Number of Animals*	PERCENT OF RABBITS SHOWING		
		Fall of Complement	*Formation of Antibody*	*Glomerular Lesions*
Controls[a]	31	95	80[d(1)]	80
X-radiation[a]	23	0	0	0
Nitrogen[a,b] mustards	14	0	7	14
Controls[c]	15	100	60[d(2)]	80
ACTH[c]	11	100	55[d(2)]	0

[a] These animals all received single injection of 1 gram per kilo of bovine serum gamma globulin.

[b] In four rabbits the dose was inadequate to produce consistent leukopenia or to prevent antibody formation; these four are omitted.

[c] These animals all received second injection of 1 gram per kilo of bovine serum gamma globulin on the tenth day and were sacrificed 48 hours later.

[d] Discrepancy with figures in Table 1 is due to (1) earlier sacrifice of animals; (2) second injection of antigen before antibodies were demonstrable in certain animals.

The cause of the inflammatory reaction excited by the union of antigen and antibody in the tissues is obscure. It has been suggested that complement, with its lytic effect on sensitized red blood cells, might lead to the lysis of tissue cells, to which the antigen was fixed, upon their combination with antibody, and that the enzymes and various pharmacologically active substances released from such cells might mediate the inflammatory response (28). All this is purely speculative, although a similar mechanism has been demonstrated for white blood cells (31). Efforts

to find a substance which might inactivate and thus depress the level of serum complement for a sufficient period to demonstrate its essential role in the tissue reactions have all ended in failure (32).

The substances released by the union of antigen-antibody in the tissues and thus responsible for the inflammatory reaction have not been identified. Histamine can be neutralized by the antihistaminic group of drugs; in man, these drugs, the salicylates, and intravenous procaine have been used to control the symptoms of serum disease. Little has been reported on the effects of these drugs on the lesions induced by the injection of foreign proteins in experimental animals. Our own studies with benadryl (33) have not indicated that the administration of antihistaminic drugs would suppress the development of hypersensitive lesions in rabbits.

As might be expected, the literature upon the effects of adrenal corticosteroids on experimental hypersensitivity is growing rapidly. There is general agreement that either adrenal stimulation by adrenocorticotropic hormone (ACTH) or administration of cortisone will suppress the development of lesions in animals injected with foreign proteins, but less agreement as to the mechanism by which this result is brought about. One group suggests that these hormones inhibit the formation of antibodies in the rabbit (34, 35), an action which has certainly not been confirmed in other species (36). Most investigators agree that the delayed type of reaction in experimental animals can be markedly suppressed by ACTH or Cortisone—the tuberculin reaction in tuberculous animals (37), the Arthus reaction in animals receiving daily injections of horse serum (38), or egg albumin (39), and allergic encephalomyelitis (40)—while the immediate anaphylactic response (41), or the Arthus reaction in highly sensitized (active or passive) rabbits (35, 42) is not affected. Suppression of a closely related type of tissue response, the Schwartzman phenomenon, has also been reported as a result of administration of these hormones (46). The investigations of three groups (43, 44, 45) studying experimental serum sickness in rabbits are in complete agreement that ACTH can suppress the allergic lesions without interfering perceptibly with the development of antibodies and that the fall in complement, indicating the interaction of antigen with its antibody, may occur despite the administration of ACTH. It seems probable that any discrepancies between these results and those of others may be explained on the basis of differences in

experimental procedure or in quantitative factors[1] such as the concentration of steroid achieved or the level or type of antibody present.

SUMMARY

Experimental serum sickness in the rabbit has proved to be a very useful biological system for investigating some of the problems of hypersensitivity, since Rich and Gregory first demonstrated that lesions, resembling those seen in a number of human diseases presumed to be on an allergic basis, could be reproduced in rabbits by the injection of foreign serum proteins.

The pathological and serological sequences observed in rabbits following the injection of a single large dose of crystallized bovine serum albumin or of bovine serum gamma globulin differ strikingly. Because of the uniformity of response to doses of 1 gram of bovine serum gamma globulin per kilogram, this protein has been used as the antigen in a number of experimental studies.

The hypothesis has been put forward that the glomerulonephritis observed in most rabbits 8 to 12 days after the injection of this antigen is the result of the interaction of antigen fixed in the glomerular tissue with antibody to bovine serum gamma globulin and with complement, as a result of which inflammatory changes develop.

It has not been possible to demonstrate selective localization of this antigen in the glomeruli with labeled antigens, although interesting information concerning the behavior of foreign proteins in the rabbit has been obtained. The superiority of I^{131} to the azo dyes as a label for the serum proteins, because attachment of the former does not appear to alter their molecular properties and physiological behavior so drastically, has been demonstrated.

The development of glomerular lesions has been prevented by pretreatment of the animals with either X-radiation or nitrogen mustards, procedures which inhibit the formation of antibodies, thus indicating

[1] Timing and dosage are very important. In our experiments ACTH was not administered until several days after injection of the antigen, at a time when neither x-radiation nor nitrogen mustards would have had any effect on antibody formation. If hormone treatment is begun at the time of injection of the antigen, the results may well be different. Moreover, in contrasting the effects of cortisone and ACTH, it should be pointed out that higher blood and tissue levels of corticosteroids can probably be produced by the former than by ACTH stimulation.

that antibody is essential for the tissue reaction. It has not been technically possible to prove that complement is necessary in this system, although the regular depression of its titer during the period of *in vivo* antigen-antibody combination is evidence that it interacts with the antigen-antibody complex.

Stimulation of the adrenal cortex by adreno-corticotrophic hormone is effective in blocking the development of glomerular lesions without preventing the interaction of antigen, antibody, and complement and thus appears to protect against the irritant effects of this combination.

The reasons for the localization of the lesions of experimental serum sickness, the chemical characterization of the antibodies responsible for the tissue lesions, and the actual biochemical mechanism of the inflammatory response induced by the antigen-antibody reaction in the tissues remain to be elucidated.

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Chapter 13

THE LOCALIZATION OF ANTIGEN IN TISSUE CELLS BY MEANS OF FLUORESCEIN-LABELED ANTIBODY[1]

By Albert H. Coons, *Harvard Medical School*

The injection of a foreign protein is one of the basic acts of the immunologist, an act which sets in motion the train of biological events which forms the subject matter of this symposium. It is my purpose briefly to review some recent findings concerning the distribution and persistence of several antigenic materials after their intravenous injection into mice.

The fate of such injected antigens has excited the interest of immunologists from the early days of the subject, and various experimental approaches have been used in an effort to answer the simple preliminary question, Into what cell types do antigenic molecules penetrate? For example, toxic antigens such as diphtheria or tetanus toxin have been injected into chickens, rabbits, and guinea pigs, various organs subsequently removed, and portions of them ground and injected into susceptible animals to assay the toxin present (1, 2, 3). In other experiments, foreign chemical atoms such as arsenic and iodine have been chemically introduced into protein molecules, these materials injected, and various organs analyzed chemically for the foreign chemical label (4, 5). Both these methods yield quantitative data but no histological information. Another approach introduced by Sabin was to inject proteins labeled with dye molecules (6). Histological examination of the tissues could then be made for the presence of the dye. This approach was extended by Smetana and Johnson (7), Smetana (8), Kruse and McMaster (9), and Gitlin (10). McMaster has already reviewed the facts which these studies have brought to light. I will only summarize them by stating that they all agree in finding such colored proteins in greatest amount in phagocytic cells throughout the animal, and in

[1] Aided by grants from the Life Insurance Medical Research Fund, the Helen Hay Whitney Foundation and the Eugene Higgins Trust.

smaller concentration in the epithelium of the proximal convoluted tubule of the kidney, and in the cytoplasm of fibroblasts.

Antigenic molecules, however, are branded during their synthesis by their serological specificity. One would anticipate that, if a drop of antibody were placed over a tissue-section containing homologous antigen, the antibody would adhere as a microprecipitate to those cells containing the antigen. If the antibody molecule were labeled in such a way as to make it visible through the microscope, the result would be a specific histochemical stain which could be used to reveal the presence of antigen. Such a method would improve the strategic situation by allowing the study of natural antigens uncompromised by prior chemical manipulation. Although there were earlier experiments involving the diazotization of antisera (11, 12), Marrack was the first firmly to establish that antibody molecules could be coupled to a dye molecule without losing their immunological activity (13).

Preliminary experiments in our laboratory with such colored antibodies indicated that color was too weak to use as a method of identifying the minute amounts of antibody deposited over an antigen-containing cell. Fluorescence is a much more powerful phenomenon, and it was easily demonstrated that antibodies coupled through the carbamido linkage to fluorescent compounds retained their serological specificity and had a brilliant fluorescence in ultraviolet light (14, 15). Antisera prepared in rabbits against the chosen antigens were coupled through the carbamido linkage with fluorescein isocyanate. After purification, these antibody solutions were used as stains by spreading a drop of one of them over a section of the tissue to be explored, allowing time for the antibody to be precipitated over the sites containing homologous antigen, removing the unprecipitated fluorescent proteins by careful washing, and then examining the otherwise unstained tissue under the fluorescence microscope (16). Controls of the specificity of the resulting yellow-green fluorescence were as follows:

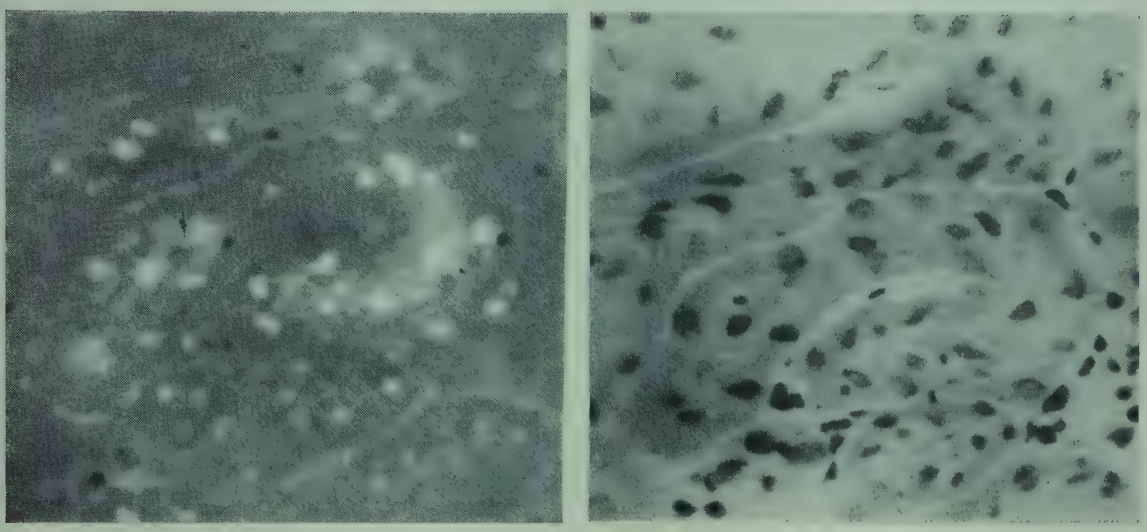
1) Failure of the fluorescent antibody solution to produce fluorescence in normal tissues;
2) Failure of heterologous labeled antibody to produce fluorescence in the experimental tissues;
3) Specific inhibition of the fluorescence of a companion slide by previous treatment with unlabeled homologous antiserum;

4) Specific removal of labeled antibody from an aliquot of the homologous labeled antibody solution, the supernatant failing to stain the experimental tissue.

This method has been successful in studies of the distribution of bacterial polysaccharides (17, 18) and proteins (19) after their intravenous injection into mice. There have also been some difficulties and some failures (16).

The data so far collected by the use of this method are the fruit of simple exploratory experiments and the data themselves are qualitative. The antigens studied to date are as follows: pneumococcal capsular polysaccharides Types II and III; Friedländer polysaccharide Type B; human plasma gamma globulin; crystalline bovine serum albumin; crystalline egg albumin. All six of these antigens appeared promptly and in high concentration in the connective tissue of all the organs examined. They were also visible rather promptly in the cells of reticulo-endothelial system where their concentration gradually increased. They were all found in low concentration in the cytoplasm of lymphocytes, in the lymphoid follicles of the spleen and lymph nodes. They all made a prompt appearance in the hepatic cells and in the cells lining the renal tubules. The pneumococcal polysaccharides were clearly demonstrable in the myocardial muscle cells, but it has not been possible to establish the presence of the other four antigens in this location.

These striking common features in the distribution of antigenic molecules of widely differing nature should not be allowed to obscure the differences in their fate which came to light during the course of these studies. For example, there was a difference in distribution among the six antigens in the kidney. The cells of the glomerular capillary loops usually contained appreciable amounts of antigenic material in the case of the bacterial polysaccharides and the larger protein molecules, but egg albumin was a striking exception because it could not be found in these cells despite the fact that it was being rapidly filtered through the glomeruli at the time the animals were sacrificed. On the other hand, egg albumin was more prominent in the cytoplasm of the cells lining the proximal convoluted tubule than were the other protein antigens, and, in contrast, all three bacterial capsular polysaccharides appeared in a narrow zone of the renal tubule, in the cytoplasm of the cells lining the juxtaglomerular portion of the distal convoluted tubule.



Convoluted Tubule of Kidney (Mouse 377; 10 mgm. Crystalline Egg Albumin Intravenously, Killed after 30 Minutes)
Antigenic material as revealed by fluorescein-labeled antibody is present in the cytoplasm and nuclei of the tubular epithelial cells (left). After the left-hand picture had been taken under the fluorescence microscope, the coverslip was removed, the section was stained with hematoxylin and eosin, and the same field photographed again in visible light (right). (From Coons, Leduc, and Kaplan, *J. Exp. Med.*, 1951, 93:173.)

The principle difference in behavior among the six antigens studied was the rate of their disappearance. Among the proteins this was correlated with molecular size, the smaller molecules disappearing more rapidly than the larger. For example, egg albumin (dose 10 mgms.) could be found only in traces after about two hours and had disappeared below the level of detection within 24 hours. A similar dose of bovine albumin disappeared more slowly and did not reach trace levels until about two days after injection. Human gamma globulin persisted still longer, being readily detectable six days after the injection of 4 mgms., and persisting longer than eight days in a 10 mgm. dose. In the case of all three proteins, the reticulo-endothelial cells and the connective tissue showed traces of antigen after it had disappeared from other locations. By contrast, the three bacterial polysaccharides were very persistent. After the injection of comparable doses (2 to 4 mgms.) they were readily detectable two or three months after injection and one of them was found in one mouse allowed to survive six months following the injection of polysaccharide. Here too, however, the persistence was longest in the connective tissue and in the reticulo-endothelial system. The relatively long persistence of pneumococcal polysaccharide was first noticed by Avery and Dubos (20), who suggested that no enzymes capable of splitting such polysaccharides were present in mammalian cells.

The factors influencing the disappearance of antigenic materials are not known with certainty. It is known that they are excreted through the kidney; in addition, the proteins are probably hydrolyzed by proteolytic enzymes present in many tissue cells. The gradual rise of antibody probably also plays a part in the disappearance or apparent disappearance of the more persistent protein antigens. The rise of antibody may mask the presence of antigen by making it undetectable by immunological methods, but the recent work of Dixon, Bukantz, and Dammin indicates that the rate of disintegration of antigenic protein molecules is increased in sensitized animals (21).

By far the most striking finding during the course of these studies was the apparent penetration of protein antigens into the nuclei of all the cell types in which they could be found at all. For example, all three protein antigens could readily be found in the hepatic cell nuclei, the nuclei of the cells lining the renal tubules (see figure) the nuclei of fibroblasts, reticulo-endothelial phagocytes, and lymphocytes. This independent finding confirms the observations of Crampton and Haurowitz

(22), who found from 13–19 percent of the radioactivity in the nuclear fraction of liver homogenates after the injection of bovine serum globulin or ovalbumin labeled with I^{131}. The full implications of this finding are obscure at present. By the methods which we have used it cannot, of course, be established that the material detected in the nuclei had retained the molecular dimensions of the native injected material. This is clear from the method used to detect the presence of the antigen; any antigenic molecular fragment large enough to produce precipitation of the labeled antibody would thereby reveal its presence. By the same token, however, the hypothetical split products must have existed in the nuclei in large enough pieces not only to retain their specific immunological pattern but also to produce precipitates.

Coming finally now to the bearing of these findings on the main subject of this symposium, it can only be pointed out that our data have revealed the presence of injected antigen in phagocytic cells such as Kupffer cells, in the reticulum cells lining the lymphoid and splenic sinuses, and in developing lymphocytes in the germinal centers in the spleen and lymph nodes. We have no data concerning plasma cells. It is clear that these data do not allow a choice to be made among the cell types heretofore suspected of being the sites of antibody production. In this connection it should perhaps be emphasized that the antigens which we injected were in the form of clear solutions which had been filtered or centrifuged free from particles and that the phagocytic function of the reticulo-endothelial system is not limited to particles visible under the light microscope.

It is our hope that future studies with this and other methods may throw more light on various aspects of the physiology of antibody production, such as the influence of adjuvants on the cell types into which injected antigen finds its way. It may be possible to reverse or otherwise modify this procedure so that antibody can be detected.

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Chapter 14

RADIOACTIVE TRACERS IN THE STUDY OF ANTI-TISSUE ANTIBODY

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One aspect of anti-tissue antibodies which has been investigated from time to time since the turn of the century (1) is the use of antisera prepared against malignant tissue in the treatment of cancer. Since antisera against infectious agents have been found effective in combating the corresponding diseases, it was reasoned that antisera prepared against cancerous tissue might be effective in combating cancer.

The sera were produced by injecting tissue homogenates or fractions of tissue into rabbits. The rabbits were then bled and the serum thus obtained used therapeutically. Unfortunately, the results obtained by the original investigators were equivocal. The failures to obtain positive results may have been due to one or more reasons: (*a*) The sera may have contained no antibodies which would react with any part of the tissue. This is unlikely, since antibodies of some sort can usually be obtained by such a procedure. (*b*) The sera may have contained antibodies which cross reacted with other tissues and were completely taken up by the other tissues. (*c*) The sera may have contained specific antibodies for components inside the tumor cells, but could not reach these intracellular components and affect them. (*d*) The sera may have contained antibodies which did localize in the tissue, but did not produce an observable physiological effect, and therefore negative results were recorded.

Actually, with transplanted tumors, it has been shown by several investigators (2–6) that exposure of transplantable tumor tissue to homologous antisera before transplanting destroyed the ability of tumor tissue to propagate, and, here, we have evidence of a physiological effect of anti-tumor sera.

In a reinvestigation of the field of tumor immunology, it seems reasonable that first it must be proven that there is something in an antiserum prepared against a malignant tissue which actually goes to the tissue

in question. This can be accomplished by the use of radioactive tracers. If an antiserum against the tissue is prepared and the antibodies are tagged with a radioactive element, disposition of the antibody, after its injection, can be traced by following the disposition of radioactivity. Further, it is not impossible that, if antibodies can be found which are specifically localized in tumor tissue, they can be made to carry therapeutically active amounts of radioactivity to the tissue. Thus, reliance need not be placed upon the capacity of the antibodies themselves to produce a therapeutic effect.

Another aspect of anti-tissue antibodies relates to certain diseases which appear to have an etiology of autoimmunization in which the mechanism of the disease process is a production by the individual of antibodies against his own tissues and a subsequent reaction of these antibodies with the tissues. Diseases for which such a mechanism has been postulated include glomerulonephritis, multiple sclerosis, rheumatic heart disease, periarteritis nodosa, lupus erythrematosis, and certain anemias. In these diseases it would be of value to demonstrate that antibodies are formed which do localize in the tissue involved in the disease process.

In our studies, we have shown that it is possible to label antibodies with radioactive tracers without destroying their antibody activity, and we have shown that it is possible to trace the localization of anti-kidney antibodies in the kidney and the localization of anti-lung antibodies in the lung (7, 8, 9). We have also demonstrated immunological differences between kidney, lung and liver (10).

THE LABELING OF ANTIBODIES

Non-radioactive Labels. The fact that various substances can be coupled to antibodies without destroying the ability of the latter to combine with antigen has been known for some time. Thus, as early as 1930, Reiner (11) showed that arsanilic acid could be diazotized and coupled to antibodies without destroying antibody specificity. It is not too surprising that this is possible since the antibody molecule is very large, with a molecular weight about 160,000. The molecule is about 200 Å long and about 40 Å wide, from physical measurements, and the antibody-specific region is probably only a small part of the surface of the molecule. Thus, all the remaining surface would be available for carrying coupled materials with no effect on the specific region. Marrack

(12) was able to couple a red dye to anti-typhoid serum, and showed that the agglutinated typhoid bacteria were colored red. The serum was labeled by coupling "R-salt" to one end of bisdiazotized benzidine, and the serum to the other.

Later, Coons, Creech, and Jones prepared a labeled anti-pneumococcus Type III serum by coupling the antiserum with beta-anthryliso-cyanate to form a serum containing beta-anthrylcarbamido groups (13). Pneumococcus Type III organisms, when agglutinated with this serum, showed a blue fluorescence. Coons has since continued this work using such labeled sera as specific stains to determine the localization of various injected antigens (14).

In all of the examples cited thus far, definite chemical bonds were formed between the antibody and the material coupled. For instance, diazotized compounds couple with the tyrosine, and perhaps with the histidine, to form an azo linkage. The isocyanates couple with free amino groups of the antibody proteins to form carbamido linkages. McClintock and Friedman "combined" uranium and Malachite Green with antibodies against pneumococcus polysaccharide, and showed localization of the serum in regions which had been heavily infiltrated with the pneumococcus polysaccharide (15).

Radioactive Iodine as a Label. For our work, we decided on the use of radio-isotopes as labels, since they can be determined quantitatively with ease. We chose to use radio-iodine, I^{131}, as a tracer, since iodine will substitute directly into the tyrosine, and probably the histidine groups of protein molecules (19). I^{131} is readily available and is easily obtained and easily counted. It has a half-life which is long enough for one to carry out experiments with a single preparation over a period of several weeks, but is short enough so that any possible contamination decays within a reasonable length of time.

The experimental procedure for radio-iodination is simple. Iodide ion is mixed with carrier-free radioactive iodide ion and is then oxidized to iodine with sodium nitrite in acid solution. The protein to be iodinated is then added. The reaction takes place according to the following equations:

$$2I^- + 2H^+ + 2HNO_2 \rightarrow I_2 + 2NO + 2H_2O$$

$$I_2 + OH^- + \underset{\text{protein}}{\overset{\text{OH}}{\bigcirc}} \rightarrow I^- \underset{\text{protein}}{\overset{\text{OH}}{\bigcirc}} + I^- + H_2O$$

The method used for determining the amount of radioactivity in a solution is simple. The solution is made alkaline with sodium hydroxide and is poured into a metal cap. The water is evaporated under an infrared lamp and the radioactivity in the solid residue determined with a Geiger counter.

Since the initial work (7), others have reported various uses of iodine-labeled antibodies (16, 17, 18).

The Iodination of the Antibodies. The iodination of antisera was first carried out by Breinl and Haurowitz (10), who showed that increasing degrees of iodination decreased the agglutinating power of horse antisera specific toward S. paratyphi A, S. paratyphi B, and Sh. paradysenteriae. We have shown that when anti-ovalbumin serum is moderately iodinated so that it still retains serological activity, the antibody molecules actually do contain iodine, since the specific precipitate formed with ovalbumin contains radioactivity (7). That the radioactivity which came down in the precipitate was attached to the antibody and did not come down non-specifically was shown by the following experiment: Anti-ovalbumin serum was iodinated with iodine containing a tracer amount of I^{131}. This iodinated antiserum was then mixed with antiserum prepared against beef serum coupled with diazotized para-aminobenzoic acid. The two antigens were added separately to different portions of the mixed antisera. Only the specific precipitate formed with ovalbumin was radioactive.

Effect of Iodination on Antibody Activity. When antibodies are iodinated extensively their activity is appreciably decreased (20, 21), since the molecule is apparently iodinated in the region of the specific site. Those molecules which have been iodinated but which retain their antibody activity are apparently those in which the iodine is attached in a region which does not contain the specific site. We have proved this to be the case by showing that the combining sites can be protected from iodination by combining them with a specific hapten group (22). For example, anti-benzenearsonate antibodies may be protected by the combination with benzenearsonate ions, as shown in Figure 1. In this case amounts of iodine can be added without effect which would otherwise suffice to destroy completely antibody activity.

Radioactive Sulfur 35 as a Label. Another radioactive label which we have used is sulfur, S^{35}. This was attached to protein molecules by preparing the p-aminobenzenesulfonic acid containing S^{35}, diazotizing

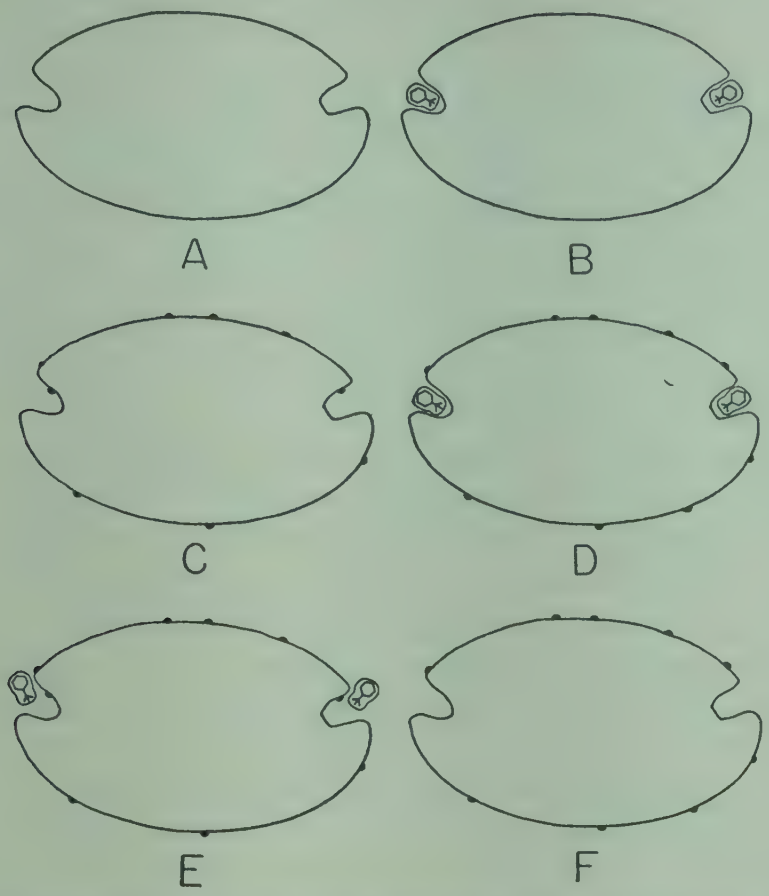
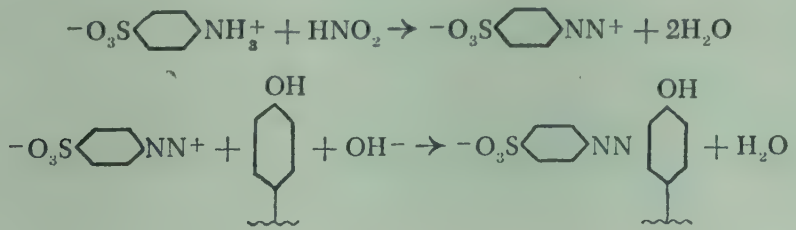


FIGURE 1. SCHEMATIC DRAWING SHOWING THE MECHANISM OF THE PROTECTION PHENOMENON

A represents the anti-p-azobenzenarsonate antibody; C, the iodinated antibody when iodination is carried out in the absence of hapten (iodine atoms are shown in black); E, the lack of reaction of this antibody with hapten; B, the uniodinated antibody combined with hapten; D, the product of the iodination of this complex; and F, the free antibody after iodination in the presence of hapten showing the unaltered combining region.

the amino group and then coupling to the protein in slightly basic solution according to the following equations.



THE LOCALIZATION OF ANTI-TISSUE ANTIBODIES

Since we have shown that antibodies can be labeled with radioactive substances, the next step was to prepare an antiserum against the tissue and to follow the localization of the antibody by the use of radioactive tracer. For this step, we chose to study antisera prepared against rat kidney. Such sera were demonstrated by Masugi (24) to be nephrotoxic and since then have been studied in several laboratories (25, 26, 27). Since this serum produces definite physiological changes in the kidney, it seemed logical that if the antibody were labeled it should be possible to follow it to the kidney.

The Localization of Anti-kidney Antibody. Antisera against rat kidney tissue were prepared in rabbits according to the method of Smadel. The globulin was separated by fractional alcohol precipitation, and subsequently iodinated with iodine containing tracer concentrations of I^{131}. Rats were injected with this radio-iodinated globulin fraction of anti-kidney serum. Control rats received a similarly prepared radio-iodinated globulin fraction of anti-ovalbumin serum. After four days the animals were sacrificed and perfused to remove any radioactivity which might have been present in the blood. Finally the tissues were analyzed and it was found that there was a definite accumulation of iodine in the kidneys of the animals which had received anti-kidney antibody. With the anti-ovalbumin serum there was practically no uptake in the kidney. Thus there is a substance, presumably antibody, present in the anti-kidney serum and absent in anti-ovalbumin serum, which localizes in the kidney. While there is some increased uptake of radioactivity in other organs when anti-kidney serum is administered, it is less than that found in the kidney. Such cross reactions are probably due to the presence of common antigens in the various organs. There remains the possibility that the localization of radio-iodine in the kidney is not directly due to the radioantibodies remaining in the kidney, but that it may be due to an ability of the anti-kidney serum to sensitize the kidney so that it can take up various substances such as radio-iodinated proteins non-specifically. This possibility, although it seemed unlikely, was investigated and was found not to be the case (28). This was demonstrated by inoculating one group of animals with a radio-iodinated anti-kidney serum, another group with radio-iodinated anti-ovalbumin serum, and a third group with a mixture of radio-iodinated

anti-ovalbumin serum and a non-radioactive, but iodinated, anti-kidney serum. Radio-iodine was taken up in the kidneys of rats of the first group, but not those in the second and third groups, showing that the iodinated anti-kidney serum did not cause an alteration of the kidney so that it would pick up the radio-ovalbumin.

We have repeated these experiments with antisera to mouse kidney and found similar localization of radioactivity in the kidneys of mice injected with the radio-iodinated anti-mouse-kidney serum (29). There appear to be no differences among several strains of mice which we have studied. Indeed, antibodies prepared against rat kidney localize in mouse kidney and antibodies prepared against mouse kidney localize in rat kidney.

Anti-kidney serum contains a good many proteins other than localizing antibody. In our experiments, we have used the globulin fraction of the serum to decrease the amount of non-specific protein injected. Nevertheless only a small part of this globulin fraction is actual localizing antibody and, since the iodine is distributed over the various protein molecules in this fraction, only a correspondingly small portion of the iodine can localize in the kidney. Moreover antibody iodinates at only one half the rate at which the whole of the globulin fraction iodinates, and thus twice as much antibody protein is localized as would appear from the radio-activity (21).

The antibody which is localized in the kidney remains for a very appreciable length of time. In the case of mice receiving iodine (8) or sulfur-labeled anti-mouse-kidney antibodies (29), the half-life of the antibody was found to be 20 days. This means that after 20 days one half of the original antibody localized in the kidney remains in the kidney and one fourth remains after 40 days. The half-life was determined by injecting mice with the radio-labeled globulin fraction from anti-kidney serum and analyzing them after various lengths of time. With the radio-iodine-labeled antibodies, the localization was followed for 54 days (almost three half-lives), and the disappearance of the antibody followed very closely a first order relationship for removal. From this relatively long half-life of the antibody in the kidney it would appear that the antibody is probably fixed to the tissues in such a way that it cannot be removed by the phagocytic cells.

We have been able to determine more precisely where the localization takes place by the use of radio-autographs (23, 30). By such

techniques we were able to show that the localization takes place in the glomerulus to a high concentration, where it appears limited to the glomerular tuft. Figure 2 shows radioautographs of sections of the kidneys of two mice, one of which had received radio-iodinated anti-kidney serum and the other radio-iodinated anti-mouse-plasma serum as a control. The same amount of radioactivity was injected in each case. The radio-autographs were prepared by exposing the tissue section to the radiographic plate and then removing the section before developing the plate. The kidney section of the animal receiving anti-ovalbumin serum shows practically no localization, whereas the section from the animal receiving anti-kidney serum shows a punctate distribution of radioactivity. By matching up the original section with the radio-autograph, it is possible to show the correspondence of these "hot spots" with the glomeruli of the section. Similar radio-autographs are shown in Figure 3 but here the section was left in place following exposure and during the development of the radiographic plate and the staining of the section. One can then match the structures in the tissue with the black regions of exposure on the radiographic plate directly underneath. It is obvious that localization takes place in the glomerular tuft and that Bowman's space is free of radioactivity, as are the tubules.

It is significant that, whereas others have found foreign proteins localized in the tubular cells and not in the glomeruli (31, 32), the localization of the anti-kidney antibodies takes place in the glomeruli and not in the tubules. Of course, some accumulation of radioactivity in the tubules was to be expected, since the rabbit serum is composed of proteins foreign to the rat and mouse, but the concentration found in the tubules was too low to affect the radiographic plate. Actually, we have been able to calculate that the concentration of the radioactivity in the glomerulus is at least thirty times the average concentration of the radioactivity in the whole kidney. Although we have succeeded in localizing an amount of antibody in glomeruli equivalent to 3 percent of the weight of the glomeruli, there was no apparent saturation even at this level (33).

We have also measured the *rate* of localization of anti-kidney antibody and found that it becomes fixed almost immediately on contact with the kidney (34). It is possibly all taken up during a single passage through the kidney. The rate of localization was determined by injecting mice with radio-iodinated anti-kidney serum, removing samples

of blood at different times and injecting the blood into recipient mice to assay for localizing radioactivity. We found that within 18 minutes all of the localizing radioactivity was removed from the circulation of the donor mouse. This represents a half-life of the localizing radioactivity in the blood stream of about five minutes. These results are in agreement with those of Sarre and Wirtz who found by an experiment involving the clamping of a renal artery that the nephrotoxic factor of an anti-kidney serum was effectively removed from circulating blood within 15 minutes (35).

Identity of Localizing Antibody and Nephrotoxic Factor. A feature of considerable interest emerges from a comparison of our results (36) with those of other workers (25, 26, 27) who have been concerned with the nephritis-producing property of anti-rat-kidney rabbit serum. Smadel (25) found that kidney-tissue homogenates effectively absorbed from nephrotoxic anti-rat-kidney rabbit serum its nephritis-producing property. We have shown that a similar homogenate absorbs the kidney-localizing radioantibody. This correspondence is further indicated by the experiments of Heymann and Lund (27) and of Solomon *et al.* (26). The former authors showed that nephrotoxic anti-kidney serum is produced by the injection of kidney cortical tissue but not by kidney medullary tissue. The latter authors demonstrated that isolated glomeruli effectively absorb the nephrotoxic factor from anti-kidney serum.

Smadel demonstrated that *liver* tissue, likewise, could absorb the nephrotoxic factor from anti-kidney serum although less effectively than does kidney itself. He showed further that the extraction of kidney tissue with organic solvents does not impair its capacity to absorb the nephritis-producing factor. Our results demonstrate an identical behavior for the kidney-localizing antibody.

The parallel between our results and those of others strongly suggests that the nephritis-producing factor and the kidney-localizing antibody of anti-kidney serum are identical.

Microscopic Localization of Anti-kidney Antibodies. We believe that the localization of the anti-kidney antibody takes place on the vascular bed of the kidney, since it is removed from the circulation so quickly (34, 35). So rapid a removal of antibody from the blood stream would require extensive, intimate contact between the blood stream and the antigen responsible for the localization. Such contact probably obtains only between the blood and the vascular endothelium, or perhaps the

vascular bed, of the organ showing specific localization. If the labeled antibody must pass through the vascular bed into the tissue spaces, and then into the extracellular circulation in the lymph prior to fixation, the process would be too slow to account for its rapid localization. It seems likely that localizing antibodies are those formed against cell-surface antigens which lie in direct contact with extracellular fluids, so that the antibody can be fixed by the cell without entering it.

Non-localizing Anti-Kidney Antibodies. Anti-kidney sera invariably contain considerable antibody directed against the soluble fraction of a kidney homogenate. However, absorption of anti-kidney sera with saline extracts of kidney fails to remove any of the localizing antibody (36) so that "titers" as determined with such extracts are meaningless. Moreover, as Smadel (25) has pointed out, there is no correlation between the precipitin titer of a serum and its cytotoxic potency. It would seem likely, therefore, that such precipitins are directed against intracellular kidney antigens, and, since antibody molecules do not pass readily into the cell, specific localization does not take place. Presumably the circulating anti-kidney antibodies which Lange *et al.* (37) find in the blood of patients suffering from glomerulonephritis are of the non-localizing type. It is probable that any actual cytotoxic antibodies present in glomerulonephritis are removed from the circulation as rapidly as they are formed.

Anti-Rat-Lung Antibodies. We have carried out similar experiments with antisera prepared in the rabbit against rat lung tissue (8, 9) and found that these sera contained antibodies which localized in the lung and also in the kidney. Kidney-localizing antibodies may be partially separated from lung-localizing antibodies by absorbing anti-lung serum with acetone-extracted kidney tissue sediment. This separation indicates that rat lung probably contains some antigen not present in kidney tissue. The properties of the kidney-localizing component of anti-lung serum are very similar to those of the kidney-localizing component of anti-kidney serums: both substances localize strikingly in glomeruli, disappear slowly from kidney, and are absorbed by that fraction of kidney homogenate which is insoluble in saline, acetone, alcohol, or ether. Thus, rat lung and rat kidney possess antigenic substances which are identical or, at least, very closely related.

Since nephrotoxic factor and kidney-localizing antibodies of anti-kidney sera appear identical and since the kidney-localizing antibodies

of anti-kidney and anti-lung sera are similar if not identical, we would expect that anti-lung sera would be nephrotoxic. Indeed, Chikamitsu (38) reported that anti-rabbit-lung serum, prepared in ducks, results in a form of glomerulonephritis in rabbits which is essentially the same as the classical experimental nephritis produced in rabbits by anti-rabbit-kidney duck serum. On the basis of his findings, Chikamitsu concluded that the nephrotoxic effects of anti-kidney and anti-lung sera are due to antibodies directed against blood vessels in general. This argument, which is supported by our results, is also consistent with the observations of Seegal and Loeb (39) regarding the production of glomerulonephritis in rats by anti-rat-placenta rabbit serum, and with Smadel's demonstration (25) that a liver homogenate can absorb the nephrotoxic factor of anti-kidney serum.

It has been proposed that human glomerulonephritis may result from conversion of kidney tissue into an antigenic form by some undefined mechanism involving a streptococcal infection with a resultant formation of anti-kidney autoantibodies. If this be so, then the foregoing considerations suggest that antibodies, capable of producing glomerulonephritis, might well arise from the interaction of streptococci with pulmonary tissue, or perhaps even with vascular connective tissue in general, rather than from the reaction with kidney tissue exclusively.

Preliminary experiments have been carried out with single preparations of anti-brain, anti-heart, and anti-liver sera, but failed to show tissue localizing antibodies (8). We would expect that it should be possible to prepare localizing antibodies against liver tissue since there are substances in liver which bind antibodies prepared against kidney and lung, indicating common or cross-reacting antigens in these tissues. However, the antiserum prepared against liver not only failed to react with the kidney or lung where cross-reacting antigens are known to be present, but also did not localize in liver as well as did anti-kidney or anti-lung sera.

Cross Reactions. We have already mentioned that there is some localization of radioantibodies from anti-kidney and anti-lung sera in organs other than the homologous one against which the antiserum was prepared, indicating that there are cross reactions between the antibodies to the various organs. In our experiments, although lung tissue did not accumulate radioactivity to as great an extent from the anti-kidney serum as from anti-lung serum, kidney tissue did accumulate

radioactivity from the anti-lung serum to a high concentration. However, the kidney-localizing material could be removed from anti-lung serum by absorption with an acetone-extracted fraction of kidney tissue homogenate (9). This experiment emphasizes that lung and kidney differ in their capacity to pick up lung- and kidney-localizing antibodies, although there appear to be similar or identical substances in the two tissues which are responsible for the cross reactions. Moreover, there are substances in the liver identical with or similar to antigens in kidney and lung, since both anti-kidney and anti-lung sera localize in the liver to a greater extent than do normal serum, anti-plasma serum, and anti-ovalbumin serum. A further example which shows that various organs may absorb different radioantibodies from anti-organ sera is the following: when radio-iodinated anti-lung antisera and radio-iodinated anti-kidney antisera were absorbed with liver tissue, the liver localizing antibodies were removed the most efficiently from each serum (9, 36).

Antibodies Purified in vivo. In order to determine more closely the differences between the antibodies fixed by the various organs from a given anti-organ serum, we have studied antibodies which had been prepared against kidney and lung homogenates and which had been purified *in vivo* from the kidney, liver, and lung (10). Rats were injected with the radio-iodinated globulin fraction of anti-kidney or anti-lung serum, and were sacrificed after one hour, an adequate length of time for localization to take place. Each organ was removed and homogenized. The insoluble sediment was washed and the radioactivity eluted with alkali. The eluates were neutralized and reinjected into rats. Control animals received eluates from the organs of animals previously injected with anti-ovalbumin serum.

In the case of the antibodies eluted from the organs of an animal which had received anti-kidney serum, it was found that the purified radioantibodies isolated from kidney, lung, and liver all localized to some extent in the kidney, but that the antibody isolated from each organ also showed some preference for localization in the organ of initial localization. Antibody isolated from kidney showed localization only in the kidney. Anti-kidney antibody isolated from liver localized in both kidney and liver. Likewise, lung-isolated anti-kidney antibody localized in both kidney and lung.

Radio-iodinated antibody from anti-rat-lung serum was similarly purified from the kidneys, liver, and lungs of donor rats, and here again the

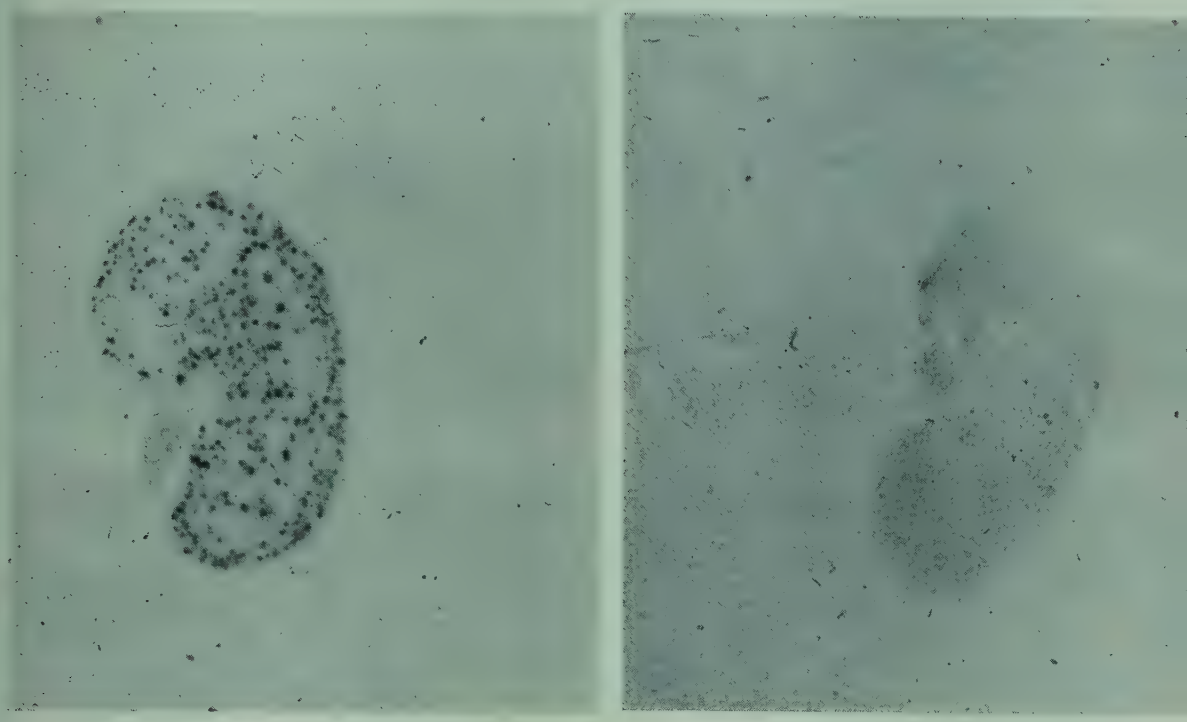


FIGURE 2. RADIOAUTOGRAPHS OF SECTIONS OF KIDNEY TISSUE
(A) from mouse injected with radio-iodine-labeled anti-mouse-kidney serum. (B) from mouse injected with radio-iodine-labeled anti-mouse-plasma serum. The intense dark spots in A are due to localized radioantibody.

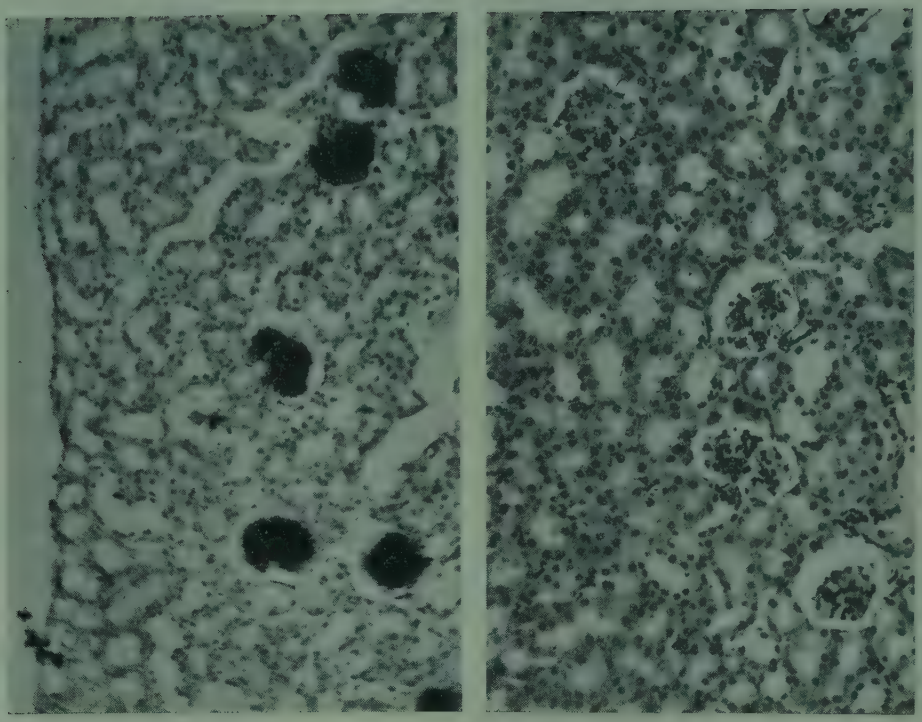


FIGURE 3. RADIOAUTOGRAPHS SHOWING THE GLOMERULAR LOCALIZATION OF RADIOACTIVITY FROM RADIOSULFUR-LABELED ANTI-MOUSE-KIDNEY SERUM (A) AS COMPARED WITH THAT FROM LABELED ANTI-OVALBUMIN SERUM (B)

The sections were stained while still in position on the radiographic plate. The intense darkening in the region of the glomerular tuft in (A) is due to localized radioactivity.

three preparations localized to some extent in the lungs of recipient rats, but the antibody isolated from each organ again showed a preference for the organ of initial localization.

In all the experiments discussed above, the localization of radioactivity from the *in vivo* purified preparations in various organs is a localization of specific antibodies. It is not a non-specific localization of radio-iodinated non-antibody protein taking place either independently or through the possible effect of organ-derived tissue-proteins obtained in the elution procedure. This is clearly shown by the anti-ovalbumin control, in which workable amounts of radioactivity were recovered from the various organs, but which showed little localization in the tissues analyzed. Therefore the specific localizations observed with the anti-kidney and anti-lung preparations must have been due to antibodies isolated from organ tissue in the *in vivo* purification.

Extent of Purification. The solution of specifically purified antibody is really purified only with respect to the proportion of radioactivity present attached to specific antibody. The proportion of antibody to total protein present is not necessarily greater, since the solution contains new protein constituents derived from tissue. The exposure to alkali during purification may denature the antibody somewhat, but it is still capable of reacting as specifically localizing antibody.

Heterogeneity of Anti-tissue Antibodies with Respect to Cross Reaction. Antisera prepared against a single organ contain localizing antibodies with different specificities. This heterogeneity may arise from the formation of antibodies against several different antigens in the original homogenate injected. A fractionation of these antibodies could then take place in another organ containing identical antigens if the entire complement of these substances were not present in the second organ. Thus the second organ would remove only antibodies reacting with the common structures and leave behind other antibodies of different specificities.

Heterogeneity of antibodies in an anti-organ serum may result not only from the stimuli of different substances, but from the production of different antibodies to a single substance. It is known that antibody to a single protein or even a single haptenic group is heterogeneous and the antibodies present have different combining powers for the homologous substances (40, 41). Thus, a substance in a cross-reacting organ which is similar to but not identical in structure with the homologous

antigen may combine strongly with some of the antibodies specific to the homologous antigen but not with others, and thus cause a fractionation of the antibody during reaction. An example of such fractionation is the reaction of duck ovalbumin with anti-hen-ovalbumin as carried out by Hooker and Boyd (42), in which the duck protein reacted with most but not all of the anti-hen antibody, while the homologous protein could still react with the remainder of the antibody. In the case of localizing antibodies, the fractionation may take place entirely through a mechanism of limited cross reactions with substances of a more or less common structure.

Nature of the Differences between Tissues. Since we have found that the antibodies of anti-kidney and anti-lung sera, eluted from liver, kidney, and lung tissue, have different properties and since the localization of these antibodies probably takes place on the vascular bed, the differences in localization probably reflect differences in the vascular beds of liver, kidney, and lung. Differences in the vascular beds of various organs might be expected from the fact that different organs have different functions which might require special properties in the vascular beds.

Possible Application of in vivo *Purified Antibodies to the Study of Active Forms of Perivascular Diseases and to the Study of Tumor Immunity.* Since we have shown that it is probably the vascular bed which is responsible for the localization of antibodies, and since the localizing antibodies and nephrotoxic factor in anti-kidney serum seem to have the same properties, it may well be that localizing antibodies are responsible for the production of certain renal diseases (glomerulonephritis or lipemic nephrosis (43)) which have been said to have as etiology, immunization of an individual against his own tissue (44, 47). Moreover, it is possible that localizing antibodies may be associated with the production of other perivascular diseases such as multiple sclerosis and rheumatic heart disease, if these actually have an etiology of auto immunization. Thus Cavelti has reported the induction of rheumatic heart disease (44, 48, 49) in rats by the injection of heart tissue together with streptococci; and Rivers, Sprunt, and Berry (50) first reported the induction of acute allergic encephalomyelitis in monkeys by the injection of brain tissue. Although these reports indicate that a mechanism involving antibody formation may be responsible for these diseases, an attempt to transmit allergic encephalomyelitis by passive transfer of serum from

an animal having the active disease failed (51), even though complement-fixing antibodies were present (52, 53). It may well be that the disease-inducing antibodies themselves are localized as rapidly as they are formed (51). Such rapid localization would be consistent with the rapid localization of antibodies in various tissues reported here. Furthermore, it is possible, in a perivascular disease, that the involvement of one organ rather than another may depend upon the specificity of the vascular bed of the tissue involved.

If it is true as postulated, that the antibodies responsible for the disease are removed from circulation as rapidly as formed, then the place to seek disease-producing antibodies is in the tissue undergoing damage. This might be done by a procedure similar to that described here, in which the damaged tissue is extracted with base to elute localized antibody, the eluate labeled with radio-iodine and then assayed for localizing antibody by injection into recipient individuals. Thus kidney tissue might be assayed for kidney-localizing antibodies in glomerulonephritis.

In the field of tumor immunology, antibodies purified *in vivo* may play an important role. It is often possible to render an animal resistant to tumor growth by previously implanting it with the tumor in such a way that this implant regresses, leaving the animal immune to the growth of subsequent implants. This immunity has been ascribed to antibody formation (54). If this immunity is actually due to antibody formation, it may be that in this case also, the necrotizing primary tumor or the stationary second tumor implant absorb antibodies as fast as they are formed. Here again, information on the nature of the immunity may be gained by assaying the tumor tissue for localizing antibodies.

The experiments described in this paper demonstrate the use of radioactive elements as tracers in determining a localization of anti-tissue antibodies in tissue at concentrations much below those which are required to produce a physiological effect. There is also the possibility of antibodies being able to carry therapeutic amounts of radioactivity to a tissue. The preparations of iodinated antibody that we have been using usually contain about 0.5 percent iodine. This means that 1 mg. of antibody protein, which can localize in the tissue, carries 5 μg of iodine and corresponds to about 100 millicuries of iodine if all the iodine is radio-iodine.

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DISCUSSION

Dr. A. S. Wiener: Antibody formation is very poor or entirely absent in the newborn. If we immunize the pregnant mother we find antibodies in the infant which are, however, acquired through passive transfer from the mother. It is of interest, I think, that passively transferred *heterologous* antibody survives far longer in the infant than in adults. From what has been said here this may be attributed to the failure on the part of the newborn to form antibodies to facilitate removal of the heterologous serum.

By determining the curve of elimination of passively acquired Rh antibodies in newborn babies, the half-life of this antibody was found to be about 30 days. The same half-life was found to apply also to other antibodies such as alpha and beta isoantibodies, diphtheria antitoxin, and bacterial antibodies. In electrophoretic studies the gamma globulin peak, which in newborns is about as high as in their mothers', decreases and reaches a minimum at the age of three months, after which it again increases. This indicates that most, if not all, of the gamma globulin of the newborn is passively acquired from the mother in the same way as antibodies. The fact that the gamma globulin peak does not decrease as rapidly as the titer of the passively ac-

quired antibodies can be attributed to the production by infants of small amounts of gamma globulin.

Dr. McMaster: I would like to ask Dr. Pressman how long his I^{131} labeled globulin remained after injection into mice.

Dr. Pressman: We have found that I^{131} labeled antiserum proteins, other than antibody, are in general removed from the circulation in the way Dr. Dixon has described. However, anti-tissue antibody localized in the kidney is no longer available for ordinary metabolic mechanisms and therefore remains for a much longer time. It is possible that a layer of anti-rabbit globulin antibody produced by the mouse will become attached to the anti-kidney antibody localized in the kidneys of these treated mice.

Dr. Haurowitz: Dr. Dixon mentioned the disappearance of *detectable* antigen. It is of great importance to know how little can be detected. How does the sensitivity of the isotopic method compare with that of fluorescence used by Dr. Coons? It can be calculated that 6 microgram per liver is equivalent to approximately 10,000–100,000 antigen molecules per cell, and I wonder whether Dr. Coons has ever calculated how much he can find by his method. Since we found antigen present in nuclei, in agreement with Dr. Coons's findings, we were interested to know if antigen was fixed to nucleoproteins; however, the nucleoprotein fraction was free of antigen.

Dr. Dixon: We have calculated that the largest amount of antigen we could miss if uniformly distributed would be a few hundred gamma in the entire rabbit. We realize that this is a lot of protein in terms of numbers of molecules, but we wish to repeat that in the last phase, antigen was disappearing at an ever *increasing* rate.

Dr. McMaster: Do you mean disappearing from the whole blood or plasma?

Dr. Dixon: We mean from the whole animal, blood and tissues.

Dr. Coons: It is difficult to estimate the sensitivity of any method because of the *non-uniform distribution* of the antigen. We could detect pneumococcal polysaccharide in the Kupffer cells of the liver 24 hours after the injection of 50 micrograms into mice, but this still means about 10,000 molecules per cell, if we assume certain cell size and *uniform* distribution.

Dr. Kabat: I would like to ask Dr. Janeway if the bovine serum globulin which he used could have contained a small amount of anti-kidney antibody?

Dr. Janeway: This is a real possibility and an important question. I am afraid I cannot answer it.

Dr. Ehrich: Rats given 20 c.c. of a potent anti-kidney serum per kilo. develop thickening of the basement membrane, urinary leakage of protein, hypercholesterolemia, and edema but no inflammation—in other words the characteristic picture of a lipoid nephrosis syndrome.

When anti-kidney serum is given rabbits to the extent of 3 c.c. serum per kilo., there occurs an inflammatory process with proliferation of endothelial and epithelial cells—the classical picture of glomerulonephritis (*intra*capillary nephritis). The difference in the pathological picture is apparently not

due to a species difference, but due rather to the dose of serum administered. In both instances there is a special structure with which the antibody reacts, that is, kidney basement membrane.

In nephritis following the intravenous injection of horse serum, we find an entirely different picture, namely cellular proliferation essentially in the intercapillary space (*inter*capillary nephritis). This nephritis is part of the phenomenon of serum sickness which affects the mesenchymal tissue of the whole body; the mesenchyma in the glomerulus is contained in the intercapillary space. We find no endothelial proliferation or thickening of the basement membrane following the injection of foreign protein. I think this answers Dr. Kabat's question.

DR. MCMASTER: There is obviously a difference in the rate of the disappearance of azo and iodinated proteins from the blood. Besides, the nature of the azo grouping may make a difference. It is also important to be certain that coupling has really been successful.

In closing, I feel that I should mention a few findings from our laboratory which are at variance with some of those reported here. This is done because I have not had an opportunity to mention these findings at this meeting and because the differences should be called to mind. We have used, in our laboratory, a method for detecting antigen that is wholly different from the usual chemical and serological ones. Blood and tissues from mice previously injected with antigen are injected intraperitoneally into normal mice, and the latter are subsequently tested for sensitivity to the absorbed antigen by challenging them with an intravenous injection of antiserum specific for the antigen to be detected. The appearance of extremely delicate ear vessel responses, visible under the microscope, signify that sensitization has occurred.

Using bovine V-globulin, as an antigen, intravenously injected into mice, we have detected antigen in the blood after 56 days, and in the liver for 101 days. Human serum albumin, injected intravenously into mice, disappeared much more rapidly, and could not be found in the blood after 23 days, nor in the liver after 36 days. The detection of antigen apparently persisting, as indicated by these tests, far longer than is generally supposed, may possibly be explained by the difference in the method used, or by its delicacy, or by the fact that the tests were carried out upon mice.

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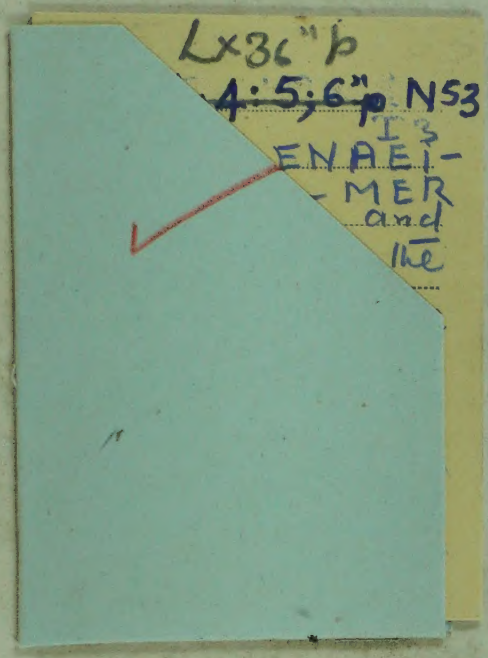
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